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Principal Investigator Name:	Jay Agarwal
Organization and Address:	University of Utah, School of Medicine Salt Lake City, UT 84132
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5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jill Shea and Jayant Agarwal

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Utah
30 North 1900 East #3B400,
Salt Lake City, UT 84132

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project is directed at improving the rate of nerve regeneration, through the development and testing of a novel nerve conduit. This project explores the potential of combing a nerve conduit that will act as an axon guide for the regenerating nerve and a drug delivery device to deliver nerve growth factor (NGF) and/or glial cell line-derived neurotrophic factor (GDNF) for at least 30 days to improve rate of nerve regeneration. We have successfully manufactured a dual chamber device, as well as further validated a diffusion model that can be used to design the nerve conduit a priori. That is by knowing what concentration of growth factor we want to deliver and for what duration we can then determine the approximate reservoir volume, diffusion hole, and growth factor concentration that need to be established to meet these design criterion. We have further determined the concentration of NGF and GDNF that need to be delivered simultaneously to result in optimal nerve growth. Finally, we have performed both in vitro and vitro studies to determine the efficacy of the device and the bioactivity of the released drugs.

15. SUBJECT TERMS

Nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Unclassified	81	19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified			

TABLE OF CONTENTS

1. INTRODUCTION:

This project is directed at improving the rate of nerve regeneration, through the development and testing of a novel nerve conduit. Our technology is a fusion between a nerve conduit and a biodegradable drug reservoir. Combat gear for the modern day warrior has greatly improved protection for the head and body, but limbs are still highly exposed to injury. Consequently, the most frequent combat nerve injuries are in the upper and lower extremities. Patients often suffer from life-long loss or functional disturbances mediated by the injured nerve, which can severely diminish their quality of life. Autologous nerve grafts serve as the state-of-the-art but numerous challenges associated with this approach results in functional benefits to only 40-50% of patients with the additional consequence of nerve-graft donor site morbidity.

This project will explore the use of combing a nerve conduit that will act as an axon guide for the regenerating nerve and a drug delivery device to deliver nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) for at least 30 days to improve the nerve regeneration. The use of PLGA or collagen has been tried previously. These devices typically focus on loading the drug in hydrogels or embedding the drug within the conduit. The shortcomings of current devices in terms of burst effect, non-uniform dosage, and uneven drug delivery, necessitates a new approach to deliver drug for nerve regeneration. The currently proposed design is advantageous, as it will allow for controlled drug release that can be made with the ability to vary the concentration, duration, and rate of release of multiple drugs.

2. KEYWORDS:

Nerve regeneration, peripheral nerve regeneration, nerve conduits, autograft, drug delivery device, nerve growth factor, glial cell line-derived neurotrophic factor, polytetrafluoroethylene, nerve grafts, axon growth, dorsal root ganglion cells, axon density, motor neuron, sensory neuron

3. ACCOMPLISHMENTS:

3.1 What were the specific objectives of the project?

Specific Aim 1 -- To optimize release kinetics of NGF and GDNF in vitro using our novel drug delivery conduit.

Tasks/Subtasks:
1. Manufacture Devices for use in 15mm nerve gap ....(Gale,)(0-10 months)
   a. Optimize PGLA ratios..(Gale/Terry)(0-4months)
   b. Optimize nanoporous membrane dimensions ....(Gale)(2-6months)
   c. Optimize reservoir dimensions ....(Gale)(4-8months)
   d. Manufacture and assemble components...........................(Gale)(6-10months)

Progress: We finished all components of Aim 1. Our final conduit was manufactured with PTFE instead of PLGA since PLGA did not maintain its structural integrity sufficiently for our device design. Specific details described in Section 3.2

Specific Aim 2 -- To evaluate the effectiveness of the conduit-drug delivery device at releasing bioactive concentrations of NGF and GDNF sufficient to enhance axon growth in dorsal root ganglion (DRG) cell culture

Tasks/Subtasks:
1. In Vitro NGF/GNDF release kinetics experiments ....(Gale, Agarwal)
   (11-18months)
2. ELISA detection of NGF/GDNF ....(Gale, Agarwal)
   (11-18months)
3. Axonal growth of DRGs ....(Terry, Shea)
   (11-18months)

Progress: We finished all three tasks for Aim 2: in vitro release, measurement of NGF/GDNF, and bioactivity. Specific details described in Section 3.2

Specific Aim 3 -- To evaluate the effectiveness of the conduit-drug delivery device to enhance nerve regeneration across a nerve gap in a mouse sciatic nerve model.

1. IACUC approval, obtain N=160 animals ....(Agarwal, Shea)
   (19-20 months)
2. Implant Device ½ devices (Mix 30-90 day groups)........................(Agarwal, Shea)
   (21-25months)
       a. ELISA for NGF/GDNF detection of day 30 animals ....(Gale)
           (24-29 months)
       b. Walking Track ....(Agarwal, Shea)
           (21-28months)
       c. Histology ....(Agarwal, Shea)
           (22-32months)
           (H&E, immunohistochemistry, retrograde labeling, muscle histology)
       d. Electrophysiology...(Clark)
           (24-30months)
3. Implant Device ½ devices (Mix 30-90 day groups)........................(Agarwal, Shea)
   (26-30months)
       a. ELISA for NGF/GDNF detection of day 30 animals ....(Gale)
           (29-33 months)
       b. Walking Track ....(Agarwal, Shea)
           (26-33months)
       c. Histology ....(Agarwal, Shea)
           (27-34months)
           (H&E, immunohistochemistry, retrograde labeling, muscle histology)
       d. Electrophysiology...(Clark)
           (29-34months)
4. Data Compiling/Analysis and Manuscript Preparation...................(Agarwal, Gale, Shea, Clark, Terry) (30-36months)

Progress: We have finished the tasks of Aim 3 except for task 4 as we are still preparing the manuscript. Specific details described in Section 3.2

3.2 What were the major activities and significant results under these goals?

<u>Aim 1: Tasks/Subtasks:</u>
Task 1. Manufacture Devices for use in nerve gap
    a. Optimize PGLA ratios
    b. Optimize nanoporous membrane dimensions
    c. Optimize reservoir dimensions
    d. Manufacture and assemble components

We manufactured our dual reservoir device with the following parameters: An outer hollow PTFE tube of 1.8mm diameter defines the drug reservoir limit (**Figure 1 and Figure 2**). A second inner PTFE tube of 0.8 mm diameter defines the guidance tube. The space between the inner and outer tubes defines the reservoir volume, which stores the drugs of choice enclosed with rubber o-rings. A laser machined hole in the inner tube is created for the reservoir chamber to define the diffusion area for drug to transport from the drug reservoir to the regenerating nerve. Our device had two approximately 120μm diameter diffusion holes.
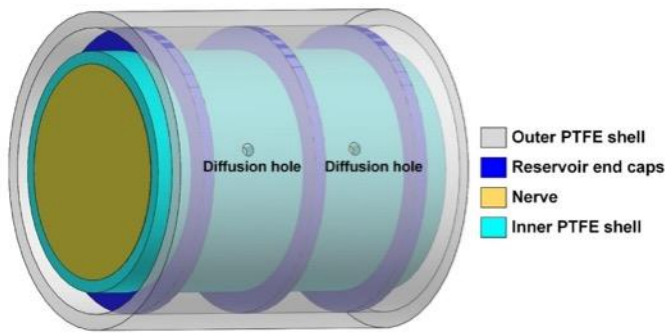
Figure 1. *Schematic of the PTFE based dual drug delivery nerve conduit*
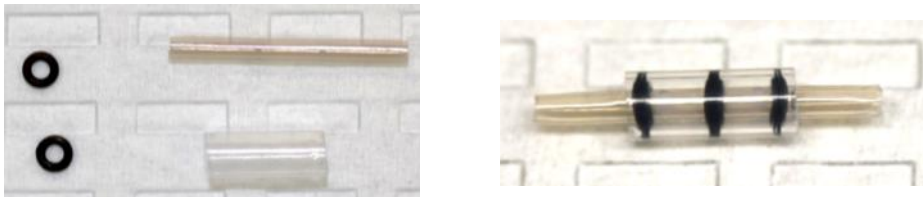

Figure 2. *(left) Conduit Components - inner conduit, outer conduit, and end caps (right top) Inner conduit with two end caps defining the outer boundary of the reservoir (right) Completed PTFE device with inner and outer tube, as well as a diffusion hole on the inner conduit that enables the release of drug from the reservoir into the inner chamber (bottom)*

Aim 2: Tasks 2-4
Task 2. In Vitro NGF/GNDF release kinetics experiments
Task 3. ELISA detection of NGF/GDNF
Task 4. Axonal growth of DRGs

Verification tests were performed to ensure that the nerve conduits released drug as expected. The loaded devices were attached to the side wall of their respective receiver chambers where tests were conducted. A 5mL transport tube was used as the receiver chamber for all of the tests conducted. PDMS was used to attach the devices to the wall in a location that would still allow for the devices to be fully submersed after the receiver chambers were filled with growth media. Samples were collected from the receiver chamber at predetermined time points. Sink method was used for drug collection. Once the fluid was collected, the receiver chamber was flushed and new medium was added to the chamber. After collection, samples were loaded into 96-well plates and amount of NGF and GDNF was determined using an enzyme-linked immunosorbent assay (ELISA) (NGF Elisa, GDNF Elisa; R&D systems, PA). The release kinetics of NGF and GDNF from our device over 30 days are depicted in **Figure 3**. The devices with no diffusion holes were loaded with both NGF and GDNF. Our device was able to co-release both NGF and GDNF for approximately 30 days. Drug loaded devices with no diffusion holes did not release either NGF or GDNF over the 30 day time frame.

The media collected on days 1, 7, 15 and 30 from the drug release tests was evaluated for bioactivity. As a reference, the DRG cells were also exposed to varying concentrations of fresh NGF+GDNF (0, 1+1 ng/mL respectively), as shown in **Figure 4**. The drug release collected from devices 1, 2 was chosen for these experiments. The released media collected from the devices exhibited similar levels of neurite growth when compared with 1+1 ng/mL of freshly prepared NGF+GDNF ($p > 0.05$). The results suggest that the drugs (NGF+GDNF) released from the devices in the period from days 1-30 promoted neurite growth in chick DRGs.
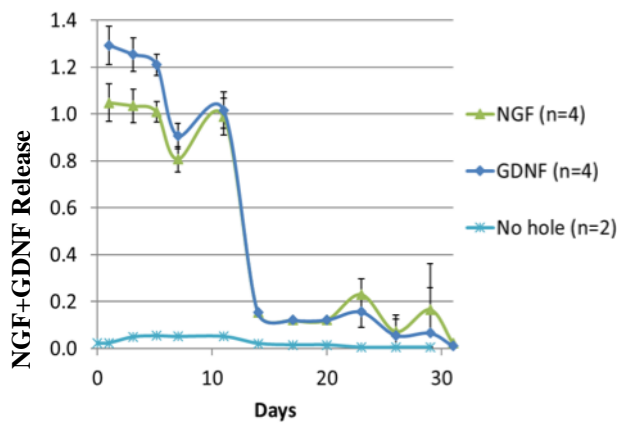
6

Figure 3. NGF and GDNF release concentration (ng/ml/day) over a period of 30 days in vitro. Plot shows average release concentration (ng/ml) for devices loaded with 0.01 mg/ml of NGF and GDNF (n=4) in separate reservoirs; device with no holes loaded with 0.01 mg/ml of NGF and GDNF. The
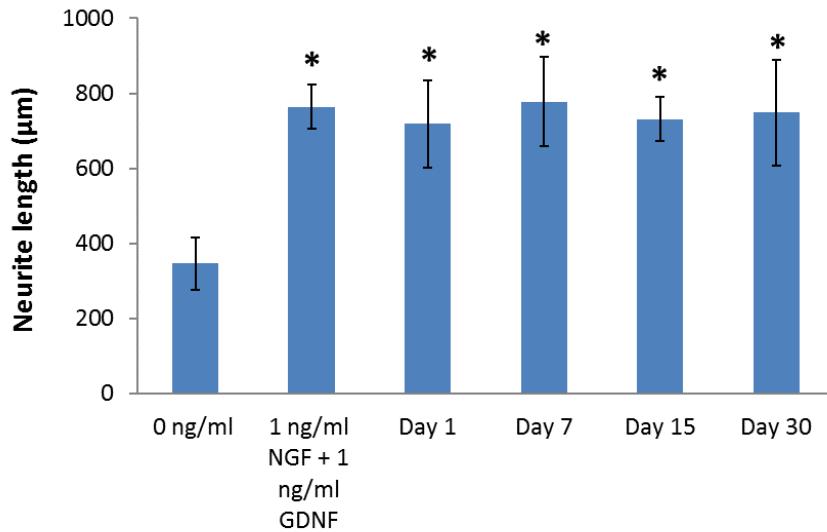

Figure 4 Bioactivity of NGF+GDNF release collected from the devices. Bar graph shows average neurite elongation (μm) of DRGs treated with NGF+GDNF released from devices 1 and 2 compared to DRGs treated with freshly prepared NGF+GDNF (Control groups).

Specific Aim 3 Tasks 5-8
Task 5. IACUC approval, obtain N=160
Task 6. Implant Device ½ devices (Mix 30-90 day groups)
     a. ELISA for NGF/GDNF detection of day 30 animals
     b. Walking Track
     c. Histology   (H&E, immunohistochemistry, retrograde labeling, muscle histology)
     d. Electrophysiology
Task 7. Implant Device ½ devices (Mix 30-90 day groups)
     a. ELISA for NGF/GDNF detection of day 30 animals
     b. Walking Track
     c. Histology   (H&E, immunohistochemistry, retrograde labeling, muscle histology)
     d. Electrophysiology
Task 8. Data Compiling/Analysis and Manuscript Preparation

GDNF Releasing Device
The *in vivo* testing of this device was designed to evaluate nerve regeneration using a mouse sciatic critical nerve gap injury model (10mm). We compared nerve regeneration between animals that had a gap injury repaired with a media filled conduit (control group) or a conduit that released GDNF. The main outcome measures were muscle atrophy, electrophysiology, motor endplate reinnervation compared over 10 weeks.

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Gastrocnemius Atrophy

The gastrocnemius muscle weight of the GDNF treated group was ~ 60% of the non-experimental side at 10 weeks. GDNF conduit group (49.4±1.4 %) had statistically less muscle atrophy than the control group (65.1±5.1 %) (p<0.05) at 10 weeks (**Figure 5**). **Figure 6** is a fluorescent image showing the FK506 releasing device with the nerve regenerated across a 9mm gap through the device and reinnervating the gastrocnemius.
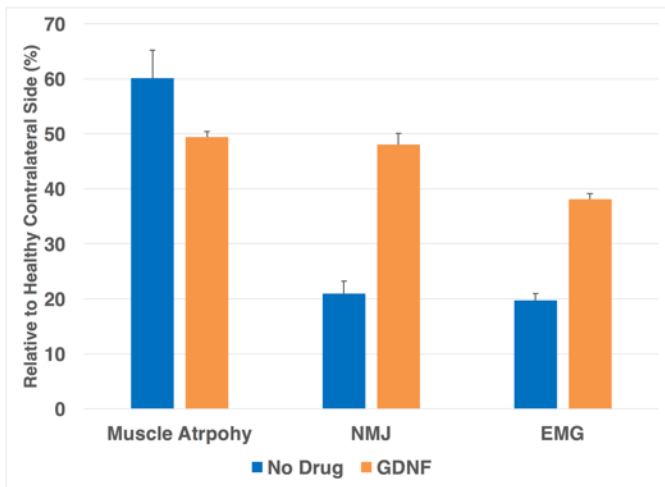

Figure 5. Sciatic nerve injuries treated with GDNF releasing devices exhibited less gastrocnemius muscle atrophy, had greater neuromuscular junction connectivity, and greater muscle compound action potentials compared to control conduit devices.
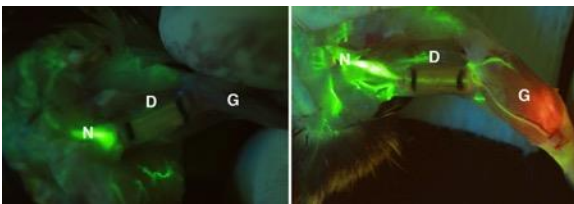

Figure 6. *Image of no drug device (left) and GDNF PTFE device (right). The GDNF releasing device had greater innervation of the gastrocnemius. (N): nerve, green in images, (D) PTFE device, and (G) gastrocnemius.*

Motor endplate evaluation

The GDNF conduit group had significantly more receptors (48.1±2 %) attached to nerve fibers compared with the control group (29.7±1.3 %) (p<0.05) at 10 weeks (**Figure 5 and Figure 7**). The neuromuscular junction data supported the muscle atrophy data as shown above.
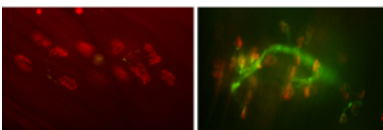

Figure 7. *Fluorescent image of neuromuscular junctions (nerve: green; acetylcholine receptors: red) for no drug control (left) and GDNF (right). There was greater connectivity in GDNF groups compared to no drug control.*

Electrophysiology

The motor activity was evaluated using electrophysiology test at 10 weeks (GDNF vs No drug). The data was reported as the percentage of motor activity (mCAP at experimental side/mCAP at non-experimental side). The GDNF conduit group had significantly more % mCAP activity (38.1±2 %) compared with the control group (23.4±1.3 %) ($p < 0.05$) at 10 weeks (**Figure 5**). The electrophysiology data supported the muscle atrophy and NMJ data as shown above.

Nerve Histomorphometry

At 10 weeks, we observed that the distal nerve in the GDNF treated group had significantly more myelination compared to No drug conduit. The no. of myelinated nerve fibers with GDNF treated device were 2141±578 compared to 155±38 with No drug treated device (**Figure 8**) ($p < 0.05$).
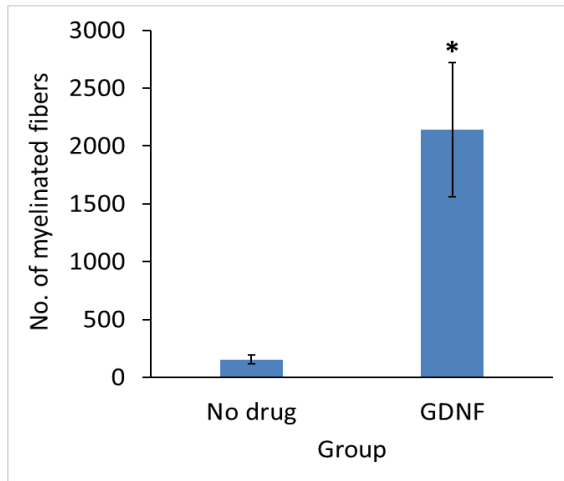

Figure 8. Nerve histomorphometry (number of myelinated fibers at the distal end) at 10 weeks for GDNF group vs. No drug group

Summary of GDNF Alone: Localized delivery of GDNF in a mouse sciatic nerve gap model with our drug delivery nerve conduit device improved muscle mass, as measured by electrophysiology and muscle atrophy, and nerve regeneration, as measured by number of myelinated fibers and neuromuscular junction connectivity.

NGF Releasing Device

We compared nerve regeneration between animals that had a gap injury(10mm) repaired with a media filled conduit (control group) or a conduit that released nerve growth factor at three different reservoir loading concentrations (1, 5, and 10ug/mL). The main outcome measures was muscle atrophy after 10 weeks of treatment.

Gastrocnemius Atrophy

We compared nerve regeneration between animals that had a gap injury repaired with a media filled conduit (control group) or a conduit that released NGF at three different concentrations. There were no differences between muscle atrophy between the media filled conduit (63±15%), NGF 1ug/mL (58±4%), NGF 5ug/mL (60±2%), and NGF 10ug/mL (54±6%) ($p > 0.05$) at 10 weeks (**Figure 9**).
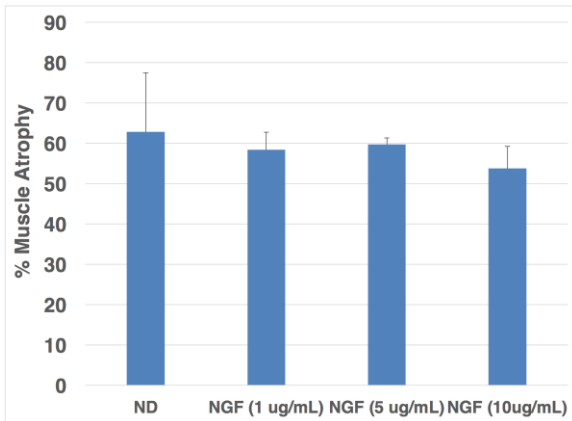
Figure 9. *Gastrocnemius muscle atrophy between mouse sciatic nerve gap injuries treats with nerve guide device alone, or NGF releasing devices. There were no group differences between the control (ND) and NGF treated groups (p>0.05).*

Summary of NGF Alone**:** There was no benefit to delivering varying concentration of NGF compare to the device alone at 10 weeks post implantation. This was in contrast with the GNDF study that demonstrated improvements to muscle atrophy at 10 weeks when GDNF was locally delivered to the repairing nerve.

NGF and GDNF Dual Release Device
The *in vivo* testing of this device was designed to evaluate nerve regeneration using a mouse sciatic critical nerve gap injury model (10mm). We compared nerve regeneration between animals that had a gap injury repaired with a media filled conduit (control group) or a conduit that released dual neurotrophic drug (NGF+GDNF). The main outcome measures were muscle atrophy, electrophysiology, motor endplate reinnervation compared over 10 weeks (NGF+ GDNF vs No drug) of treatment.

Gastrocnemius Atrophy
At 10 weeks, there were no group differences between the control group and NGF+GDNF conduit group in terms of gastrocnemius muscle atrophy (**Figure 10**). The gastrocnemius muscle weight of the treated side was approximately 45% of the non-experimental side for the no drug and NGF+GDNF treated groups.

Motor endplate evaluation
At 10 weeks we evaluated the degree of connectivity between acetylcholine receptors and nerve fibers. The data is reported as the percentage of acetylcholine receptors connected to nerve fibers. There were no significant differences in the percentage of acetylcholine receptors connected to nerve fibers between the NGF+GDNF treated or no drug treated groups at 10 weeks(**Figure 11**).

Electrophysiology
The motor activity was evaluated using electrophysiology test at 10 weeks (NGF+GDNF vs No drug). The data was reported as the percentage of motor activity (mCAP at experimental side/mCAP at non-experimental side). There were no significant differences in the %mCAP recovery between the N+G treated (~26%) or no drug treated (~23%) groups at 10 weeks (**Figure 12**). The electrophysiology data supported the muscle atrophy and neuromuscular junction connectivity data.
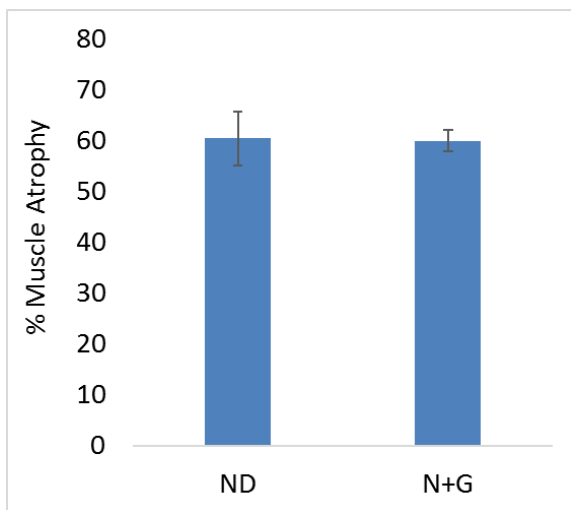
Figure 10. *Gastrocnemius muscle atrophy at 10 weeks for NGF+GDNF (N+G) vs. No drug. (ND)*
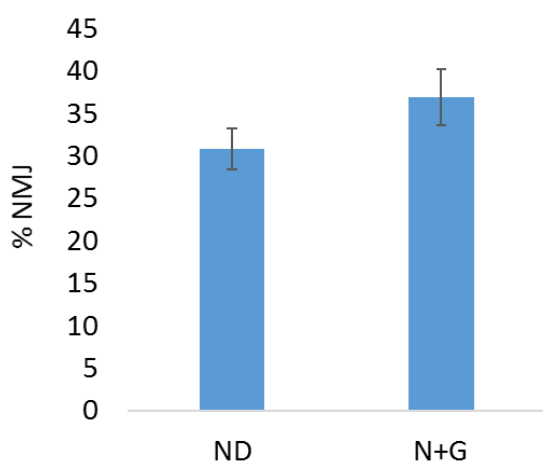

Figure 11. *Motor endplate reinnervation (%NMJ). % acetylcholine receptors reinnervated at 10 weeks for NGF+GDNF*
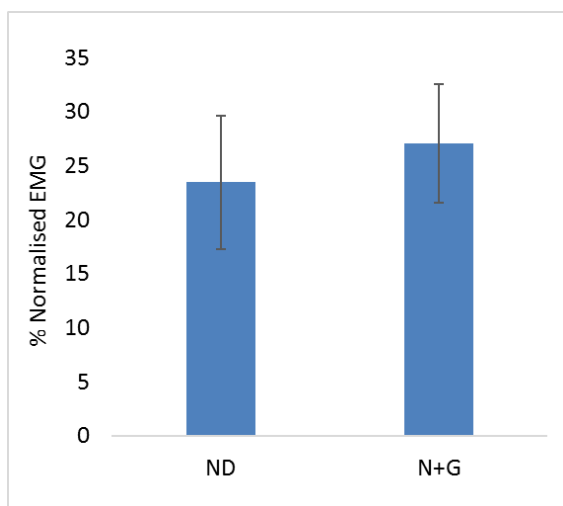

Figure 12. *% EMG activity at 10 weeks for NGF+GDNF(N+G) vs. No drug (ND).*

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<u>Summary of Dual Delivery of NGF and GDNF</u>**:** There was no benefit to co-delivering NGF and GDNF compared to a device that did not release growth factors at 10 weeks post implantation. We hypothesize the NGF was inhibiting nerve regeneration, since delivery of NGF alone or NGF combined with GDNF, did not improve muscle atrophy at 10 weeks post implantation. In contrast GDNF alone improved muscle atrophy, emg activity, neuromuscular junction connective and number of myelinated nerve fibers.

3.3 What opportunities for training and professional development has the project provided?

1. PhD research: Pratima Labroo and Brett Davis.
2. Surgical Resident research: Kyle Edwards and Andrew Simpson
3. MS research: Scott Ho.
4. Undergraduate research: Megan Roach, Rainey Cornaby, Artemis Sefandonakis, Scott Ho, and Ria Sandhu

3.4 How were the results disseminated to communities of interest?

Publication and presentation.

3.5 What do you plan to do during the next reporting period to accomplish the goals?

No further reporting periods

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

4.1 What was the impact on the development of the principal discipline(s) of the project?

One of the key impacts of the present approach is that it is possible to easily manipulate our nerve conduit device design. That is, through the use of our mathematical model it is possible to predict what size reservoir and what size hole is required to release a set amount of a drug or growth factor. This was demonstrated by our ability to quickly redesign our device from PLGA to PTFE. Without much troubleshooting we were able to release fluorescently labeled dextran from PTFE, in a manner that was consistent with our model.

4.2 What was the impact on other disciplines?

As part of this work, we have developed a new mathematical model that can be used by researchers to predict reservoir volume, drug amount, drug concentration, and diffusion hole size. This model will help researchers to avoid costly and time intensive in-vitro trials.

We have developed fabrication and sterilization protocols for a nerve conduit device with dual drug reservoirs and tested the efficacy of the device using in-vitro and DRG studies. This data will help researchers/industry to further develop drug delivery efforts in other areas as well.

4.3 What was the impact on technology transfer?

Nothing to report

4.4 What was the impact on society beyond science and technology?

Nothing to report

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

5.1 Changes in approach and reasons for change

Based on data from another project we have switched from PLGA to PTFE. The PLGA did not maintain its structure in vivo. Otherwise the device design is the same with an inner conduit, outer conduit, and diffusion hole. The transition from one material to another was also made easier with our diffusion model. Utilizing the model we were able to accurately predict the appropriate device parameters, such as reservoir size and diffusion hole, required to release NGF and GDNF.

5.2 Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report

5.3 Changes that had a significant impact on expenditures

Nothing to report

5.4. Significant changes in use or care of human subjects

Nothing to report

5.5 Significant changes in use or care of vertebrate animals.

Nothing to report

5.6 Significant changes in use of biohazards and/or select agents

Nothing to report

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.
  1. Labroo P, Shea JE, Sant H, Gale B, Agarwal J. Effect of combining FK506 and neurotrophins on neurite branching and elongation. *Muscle Nerve.* 2016 Aug 9; doi: 10.1002/mus.25370. Acknowledged federal support.
  2. Labroo P, Shea JE, Edwards K, Ho S, Davis B, Sant HJ, Goodwin I, Gale BK, and Agarwal. Novel drug delivery conduit for peripheral nerve regeneration. *J Neural Eng.* 2017 14(6):066011. doi: 10.1088/1741-2552/aa867d. Acknowledged federal support.
  3. Labroo P, Ho S, Sant H, Shea J, Gale BK, Agarwal J. Controlled Delivery of FK506 to Improve Nerve Regeneration. *Shock.* 2016 Sep;46(3 Suppl 1):154-159. Epub 2016/04/09. doi: 10.1097/SHK.0000000000000628. Acknowledged federal support.
  4. Lin KM, Shea J, Gale BK, Sant H, Larrabee P, Agarwal J. Nerve growth factor released from a novel PLGA nerve condiut can improve axon growth. Journal of Micromechanics and Microengineering 2016;26:045016. Acknowledged federal support.

Books or other non-periodical, one-time publications.

Keng-Min Lin, IMPLANTABLE DEVICES FOR SENSING AND DRUG DELIVERY IN OPHTHALMOLOGY AND RECONSTRUCTIVE SURGERY, Ph. D. Dissertation, Department of Mechanical Engineering, University of Utah, May 2014, acknowledgement of federal support

Labroo P, CONDUIT BASED DRUG DELIVERY DEVICE FOR PERIPHERAL NERVE REGENERATION, Ph.D. Dissertation, Department of Mechanical Engineering, University of Utah, May 2017, Acknowledged federal support.

Other publications, conference papers, and presentations.

Pratima Labroo, MS, Isak Goodwin, MD, Brett Davis, BS, Kyle Edwards, MD, Scott Ho, MS, Himanshu J. Sant, PhD, Bruce K. Gale, PhD, Jill E. Shea, PhD, and Jayant Agarwal, MD, Effect Of NGF Delivering Conduit On Peripheral Nerve Regeneration Plast Reconstr Surg Glob Open. 2017 Apr; 5(4 Suppl): 97-98.

Pratima Labroo, MS, Jill E. Shea, PhD, Brett Davis, BS, David Hilgart, BS, Christopher Lambert, BS, Himanshu Sant, PhD, Bruce Gale, PhD, and Jayant Agarwal, MD, FK506 Delivering Conduit For Peripheral Nerve RegenerationPlast Reconstr Surg Glob Open. 2017 Apr; 5(4 Suppl): 99.

Pratima Labroo, Jill E Shea, Himanshu Sant, Bruce Gale, and Jayant Agarwal, Controlled Delivery of Growth Factors and Small Molecules for Peripheral Nerve Regeneration, oral presentation at 2015 AIChE Annual Meeting November 10, 2015.

Pratima Labroo, Isak Goodwin, Brett Davis, Kyle Edwards, Scott Ho, Himanshu Sant, Bruce Gale, Jill Shea, Jay Agarwal, "Effect Of NGF Delivering Conduit On Peripheral Nerve Regeneration," in *Proc. of BMES 2016*, Minneapolis, MN, October 5-8, 2016.

Pratima Labroo, Himanshu Sant, Scott Ho, Bruce Gale, Jill E. Shea and Jayant Agarwal, "Controlled Delivery of Growth Factors and Small Molecules for Peripheral Nerve Regeneration," in *Proc. Of AICHE*, November 10, 2015, Salt Lake City, UT, Paper 437560, 2015.

Scott Ho, Pratima Labroo, Keng-Min Lin, Himanshu Sant, Jill Shea, Jay Agarwal, Bruce Gale, "Bioresorbable Multi-Drug Delivery Conduit to Promote Peripheral Nerve Regeneration," in *Proc. of BMES 2014*, San Antonio, TX, October 22-25, 2014.

Keng-Min Lin, Bruce Gale, Himanshu Sant, Jill Shea, William Sanders, Christi Terry and Jay Agarwal. "BSA-Filled PLGA nerve conduits for potential applications in nerve regeneration," in *Proc. of The BMES 2013 Annual Meeting*, Seattle, Washington, USA, September, 2013.

Keng-Min Lin, Himanshu J. Sant, Jayant Agarwal and Bruce K. Gale, "New approaches to bridge nerve gaps: Development of a novel drug-delivering nerve conduit," *34th Annual International Conference of the IEEE Engineering in Medicine & Biology Society (IEEE EMBS)*. August, 2012. Paper WeB 15.7

- **Website(s) or other Internet site(s)**
-
  http://www.mems.utah.edu/publications/
  This website lists the publications and research originating from Co-PI Dr. Gale's lab.

- **Technologies or techniques**

  Fabrication of biodegradable drug delivery prototypes using PLGA/PTFE. We will publish journal articles to share the device fabrication techniques.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  1. SANT HIMANSHU JAYANT, GALE BRUCE KENT, AGARWAL JAYANT P, LIN KENG-MIN, METHODS AND DEVICES FOR CONNECTING NERVES, Last status change:2013-05-10/ Fill date:2012-10-16, WO 2013066619

- **Other Products**
  1. Mathematical model based on Fick's diffusion law
  2. Fabrication of dual chamber combined PLGA/PTFE nerve guide and drug delivery device prototypes

3. Use of laser to create diffusion hole

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Personnel	Role	Percent Effort	Months
Jay Agarwal	PD/PI	8.33	1.0
Overall management of the project, guidance to students, weekly meetings and report preparation.			
Bruce Gale	Co-I	7.83	1.0
Device manufacturing, weekly meetings			
Jill Shea	Co-I	16.5	2.0
IACUC/ACURO approvals, DRG studies, ELISA, histology, animal studies, weekly meetings.			
Himanshu Sant	Co-I	21.16	2.5
Device manufacturing and validation, mathematical model, weekly meetings and report preparation.			
Christi Terry	Co-I	13.5	1.5
DRG studies, ELISA optimization			
Gregory Clark	Co-I	5.25	1.0
Electrophysiology protocol preparation			
Brett Davis	Student	50.00	6.0
DRG cell culture, ELISA, animal procedures, weekly meetings			
PLGA dual chamber prototype fabrication, mathematical model, weekly meetings.			
Pratima Labroo	Student	50.00	6.0
DRG studies, ELISA, animal studies, and weekly meetings.			

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

None

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

None

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

Attached

9. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

A Novel Drug Delivery Device for Peripheral Nerve Regeneration

OR120181
W81XWH-13-1-0363

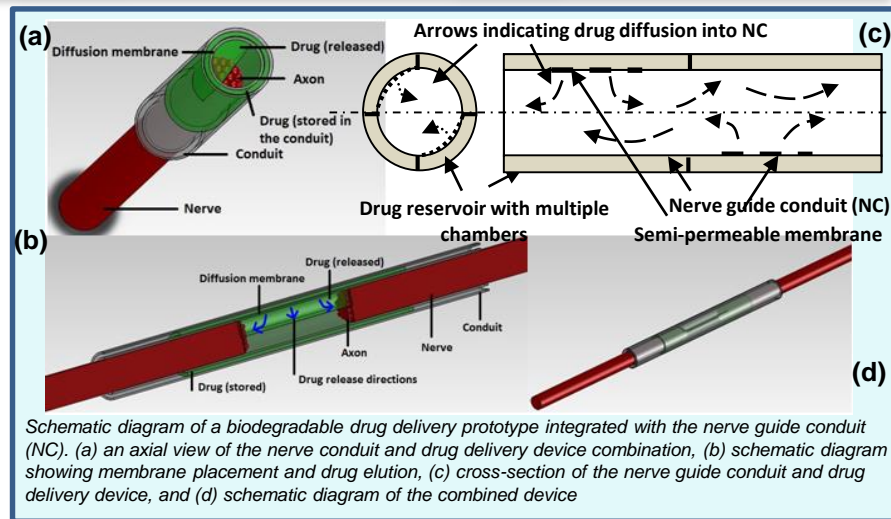**PI:** Jay Agarwal   **Org:** University of Utah   **Award Amount: Direct** $499,972; **Total**$744,958

Study Aims

• To optimize release kinetics of NGF and GDNF in vitro using our novel drug delivery conduit.
• To evaluate the effectiveness of the conduit-drug delivery device at releasing bioactive concentrations of NGF and GDNF sufficient to enhance axon growth in Dorsal Root Ganglion (DRG) cell culture.
• To evaluate the effectiveness of the conduit-drug delivery device to enhance nerve regeneration across a nerve gap in a mouse sciatic nerve model.

Approach

This proposal will explore the use of PTFE (polytetrafluorethylene) nerve guide conduits with a permeable wall and a hollow annulus that will act as an axon guide for the regenerating nerve. In addition, a drug delivery device will be incorporated with the PTFE conduit in a concentric fashion to deliver nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) for at least 30 days to improve the rate of nerve regeneration

Schematic diagram of a biodegradable drug delivery prototype integrated with the nerve guide conduit (NC). (a) an axial view of the nerve conduit and drug delivery device combination, (b) schematic diagram showing membrane placement and drug elution, (c) cross-section of the nerve guide conduit and drug delivery device, and (d) schematic diagram of the combined device

Accomplishment: We have commenced animal studies with implantation in GFP mice. Both autograft and device studies are underway.

Timeline and Cost

	Activities CY	Year 1	Year 2	Year 3
Aim 1	Mfg. of the drug delivery device-nerve guide conduit prototypes for 10 mm gap COMPLETED	▮		
	Optimization of semi-permeable membrane, COMPLETED	▮		
	Confirmation of near-zero-order NGF & GDNF release kinetics in vitro. COMPLETED	▮		
Aim 2	Testing Bioactivity of NGF & GDNF in with DRG cells. COMPLETED.		▮	
	Verification of axonal outgrowth in DRG cells with eluate of the device. COMPLETED		▮	
	Data analysis COMPLETED		▮	
Aim 3	Animal testing of novel drug delivery device COMPLETED			▮
	Data analysis COMPLETED			▮
	Report COMPLETED			▮
Estimated Budget ($)		242,766.00	247,246.00	254,946.00

Goals/Milestones

CY Year 1 Goal – Optimize release kinetics of NGF & GDNF *in vitro*
☑ Prototype fabrication and reservoir optimization: conduit dimensions
☑ Filter optimization: porosity, pore size and dimensions
☑ Optimization of NGF & GDNF and PVA concentrations

CY Year 2 Goal – Determine growth factor bioactivity with Dorsal Root Ganglion (DRG) cell culture
☑ Testing with growth factors at varying concentrations, media control and fresh aliquots for NGF and GDNF separately and in concert.

CY Year 3 Goal – Determine device efficacy with mouse sciatic nerve
☑ Implant drug delivery device and compare with empty nerve conduit
☑ Complete walking track, tissue analysis, electrophysiology and histochemistry

Comments/Challenges/Issues/Concerns None
Budget Expenditure to Date: Spent in full.

Updated: December 23, 2017

EFFECT OF COMBINING FK506 AND NEUROTROPHINS ON NEURITE BRANCHING AND ELONGATION

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Abstract

Introduction—There is a clinical need to improve the outcomes of peripheral nerve regeneration and repair after injury. In addition to its immunosuppressive effects, FK506 (tacrolimus) has been shown to have neuroregenerative properties. To determine biologically relevant local FK506 and growth factor concentrations, we performed an *in vitro* bioassay using dorsal root ganglion (DRG) from chicken embryos.

Methods—Neurite elongation and neurite branching were analyzed microscopically after addition of FK506, glial cell line-derived neurotrophic factor (GDNF), and nerve growth factor (NGF), each alone and in combination.

Results—FK506 induced modest neurite elongation (~500–800 μm) without improving neurite branching significantly. The combination of FK506 with NGF, GDNF, or both, exerted a potentiating or competitive effect on neurite elongation (~700–1100 μm) based on dosage and competitive effect on neurite branching (~0.2–0.4).

Conclusions—These results strongly suggest that the interaction of FK506 with GDNF and NGF mediates distinct enhancement of neurite growth.

Keywords

Peripheral nerve injuries have been a clinical challenge over the years.[1] The extensive network of nerve tissue throughout the body makes peripheral nerves prone to external trauma.[2] Peripheral nerve injuries frequently produce weakness, chronic pain, and neuropathies that lead to severe disability.[3] Nerve autograft has been the gold standard for treatment of injuries that result in a nerve gap.[1] Autograft involves harvesting donor nerve tissue and implanting the graft across the nerve gap.[3] Nerve autografts have been comparatively successful, but there are problems associated with donor tissue availability,[2] obtaining a donor nerve by means of single or multiple surgeries, multiple scarring sites, neuroma formation, and limited success at repairing gaps larger than 10 mm.[2] These issues

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led to fabrication of hollow, tube-like nerve guiding conduits to span gaps in nerves and provide structural support for the transected nerve.[4] These nerve guides, although structurally helpful, do not effectively replicate a nerve autograft in terms of support cells and neurotrophism and thus are not as effective at repairing large nerve gaps.[4] Following a peripheral nerve injury, neurites will tend to regrow and cross short gaps, but they need guidance and the ability to overcome the scarring process.[5]

To improve the repair process, in addition to providing guidance, therapeutic agents need to be provided at the site of transection or injury.[6] Several drugs and growth factors have been shown to be effective in enhancing neurite outgrowth across nerve gaps.[6] A study of nerve growth factor (NGF)-loaded microspheres has shown potential for repair of nerve gaps.[7] Collagen tubes loaded with NGF alone or in combination with glial cell line-derived neurotrophic factor (GDNF) have produced neurite elongation *in vitro*.[8] Use of NGF or GDNF within a specified concentration range has resulted in improved neurite growth in chick dorsal root ganglia (DRG).[9] Several papers suggest that improved peripheral nerve regeneration can be expected by supplying neurotrophins.[5,10]

The data obtained from several *in vivo* studies have indicated that NGF and GDNF synthesis is also upregulated during an inflammatory process.[11,12] After a peripheral nerve injury, proliferating and reactive Schwann cells produce growth factors, cytokines, and growth-associated proteins, which play key roles in axon regeneration and nerve repair.[13,14] It has been observed that exogenously administered NGF and GDNF increase both the number and myelination of regenerating axons.[15,16] This is due to effects of NGF and GDNF signaling both on regenerating nerve fibers and on Schwann cells and inflammatory cells,[17] and Schwann cell migration is thought to precede and promote axon elongation into repair sites.[17]

FK506 was approved by the FDA in 1994 for liver transplants and is one of the main systemic immunosuppressants used to prevent nerve allograft rejection.[18] It has also been observed to have neuroregenerative properties when administered after peripheral nerve injuries.[19,20] It can enhance the activity of NGF by increasing the sensitivity of cells toward smaller concentrations of the growth factor.[21,22] The complex of FK506 with FK506-binding protein-12 inhibits the phosphatase activity of calcineurin, resulting in accumulation of phosphorylated substrates, including nuclear factor of activated T-cells. This phosphorylated nuclear factor of activated T-cells functions as a regulator of the transcription of numerous genes, including interleukin-2 and, therefore, induces the immunosuppressive effects of FK506.[23]

There is increasing evidence that the beneficial effects of FK506 on neuroregeneration are unrelated to calcineurin inhibition and thus immunosuppression.[19,21] This first became evident as studies began to demonstrate that cyclosporine A, another immunosuppressant that works through calcineurin inhibition, does not have the same neuroregenerative properties.[23] Several research groups have demonstrated that nonimmunosuppressive FK506-binding protein ligands lack the ability to bind calcineurin but promote neurite growth *in vitro* and stimulate regeneration of peripheral nerves in rats.[24] Some studies have suggested that the neuro-regenerative effects of FK506 might result from interactions with

FK506-binding protein 52.[25] While several *in vivo* small animal studies have demonstrated improved rates of nerve regeneration with systemic FK506,[26–30] not many detailed studies have confirmed the dosage activity profile of FK506 treatment alone or in combination with other growth factors. For this purpose, we have developed an *in vitro* assay using chicken embryonic DRGs which exhibit similarities to other animal neuronal systems.

The study we report here also assessed the regulation of protein kinase B (Akt) and phosphorylated Akt (pAkt) in the process of neurite growth after single and combined treatment with FK506 and neurotrophins. Akt is a multifunctional regulator of cell survival, growth, and glucose metabolism.[31–33] It functions as a major downstream target of phosphatidylinositol 3-kinase (PI3-K), and the PI3-K pathway is involved in nerve growth factor-dependent neuronal survival.[33–35] Data suggest that FK506 treatment enhances nerve growth through activation of the Ras/Raf/MAP kinase signaling pathway downstream of PI3K-Akt.[28] GDNF also activates the Ras/MAP kinase and PI3K/Akt pathways.[36] The goal of this study was to determine how neurite growth is affected by FK506, NGF, GDNF, and the combinations of FK506 with either NGF or GDNF or both in terms of neurite length and branching.

MATERIALS AND METHODS

DRG-Explant Cultures

Fertilized chicken eggs (Merrills Poultry, Paul, Idaho) were incubated at ~39 °C under 100% relative humidity for 12 days. The eggs were first cleaned with 70% ethanol and then opened to collect the embryos. DRGs were dissected from the embryos under a stereomicroscope using a standard dissection procedure.[37] They were separated carefully from connective tissue for culturing in 24-well plates coated with laminin (1 μg/ml). Dulbecco Modified Eagle Medium (DMEM) F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% Antimycotic/Antibiotic solution were added to each well. DRGs were plated at a density of 1 per well, and growth factors were added as specified below. Cultures were maintained in a humid atmosphere at 37 °C and 5% CO_2 for 72 h. Unless specified otherwise, all reagents for cell culture were purchased from Fisher Scientific (Pittsburgh, Pennsylvania).

Drug Dosing

For each experimental condition including control, 4 DRGs of similar size were used, and the experiments were repeated 3 to 4 times. In the single growth factor experiments, the DRGs were treated with increasing concentrations of either NGF (Sigma Aldrich, St. Louis, Missouri) (0.1, 1, 5, 10, 100 ng/ml), GDNF (R&D systems, Minneapolis, Minnesota) (0.1, 1, 5, 10, 100 ng/ml), or FK506 (Astellas Pharma, Northbrook, Illinois) (1, 5, 10, 20, 50 ng/ml). For control, DRG-explants were incubated in cell culture medium matrix (DMEM+10% FBS+1% Antimycotic) without any growth factor or drug. In the experiments using combined growth factors (NGF/GDNF, NGF/FK506, GDNF /FK506, and NGF/GDNF/ FK506), the DRGs were treated with random dosages chosen using the statistical software Minitab 17. All possible combinations for each group were subjected to a fractional factorial

design in the software, which both randomly and systematically selected dosages that were tested to predict the response of remaining combinations.[38]

We used a fractional factorial design, because for experiments with many factors and levels, full factorial designs can lead to large amounts of data. Fractional factorial designs use a fraction of the runs required by full factorial designs. A subset of experimental treatments is selected based on an evaluation (or assumption) of which factors and interactions have the most significant effects.[39] The neurotrophic factor dilutions were prepared with the cell culture medium matrix (DMEM+10% FBS+1% Antimycotic).

Quantification of Neurite Outgrowth: Neurite Length and Branching

After 72 h in culture, DRGs were fixed in methanol, imaged using a widefield microscope (Nikon Spinning disk) with a phase contrast lens, and images were captured with a digital camera at $4 \times$ magnification. Phase contrast images of DRGs were taken after 3 days of incubation, and the average neurite length and branching were measured.

Neurite length and branching measurements were done using a previously discussed procedure.[9] The area of the ganglion body (A_{DRG}) and the total area of the DRG with the growing neurites (A_{tot}) were measured using ImageJ 1.31v software (National Institutes of Health, Bethesda, USA). Average neurite length (l_{avg}) was calculated by: $l_{avg} = (A_{tot}/\pi)^{1/2} - (A_{DRG}/\pi)^{1/2}$. Neurite branching was calculated from the area occupied by the neurites ($A_{neurite}$), which was determined using the threshold function (range of 100 ± 25 to 230 ± 25 pixels) available in ImageJ. Neurite branching = $A_{neurite}/(A_{tot} - A_{DRG})$

DRG Neurite Growth Rate

Images of DRGs were taken every 24 h during 3 days of incubation, and average neurite length was measured at each time point as described above. The cultured explants were not fixed for the growth rate study. From these measurements, the average neurite growth rate was determined.

DRG Lysate and Western Blot

Six DRGS were plated per well in a 6-well plate and treated with different single and combined dosages for 72 h. Cells were then lysed in Cell Extraction Buffer (Bio-source) according to the manufacturer's protocol. Cell lysates suspended in Laemmli sample buffer (Bio-Rad, Hercules, California) were heated at 95 °C for 5 min, separated by SDS-PAGE gel (Bio-Rad), and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Massachusetts). Membranes were incubated with the following primary antibodies: AKT (1:1,000, Cell Signaling Technology) and pAKT (1:2,000, Cell Signaling Technology). After washing with phosphate buffered saline (PBS) containing 0.1% Tween, membranes were incubated with a 1:5,000 dilution of horseradish peroxidase conjugated Anti-Rabbit secondary antibody (Cell Signaling Technology). Antibody was detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Massachusetts). To control for protein loading, membranes were stripped and probed with a 1:5,000 dilution of mouse anti-GAPDH (Novus Biologicals, Littleton, Colorado). Scanned autoradiographic blot images were quantified using the NIH ImageJ densitometry software.

Immunocytochemistry and Fluorescence Microscopy

DRGs were cultured for 72 h and then fixed with methanol for 10 min. Cells were washed with PBS, incubated with PBS containing Triton X-100, treated with blocking buffer containing 1% BSA (bovine serum albumin) at room temperature for 30 min, and incubated with primary antibody (β tubulin 1:200 dilution in 1% BSA in PBS-Triton X100) overnight at 4 °C. Cells were then washed and incubated for 1 h at room temperature with a secondary antibody (Rabbit 568 red 1:500 dilution in 1% BSA). Image acquisition was accomplished using Nikon Spinning disk widefield microscope and NIS Elements software.

Statistical Analysis

The data were analyzed using a multivariable linear regression model in StataIC 13. The outcome variable was neurite length and neurite branching. Specific doses of a given drug or drug combination were included in the model as indicator variables rather than assuming a specific form of dose-response, such as a linear increase across the range of doses. Interaction terms were used to test if the effects of drug combinations were greater than the sum of the effects of individual drugs. Comparisons between specific drug–dose combinations were made by varying the referent drug–dose combination in the included indicator variables, or using Wald posttest comparisons, where either approach produced comparisons analogous to independent sample t-tests. All reported P-values are for 2-sided comparisons. P-values 0.05 were considered significant.

In our experiments, the goal was to identify the drug(s)-dose(s) combination that provided the greatest response or to observe some range of effective doses. Statistical comparisons are provided merely descriptively to indicate where the maximum or plateau of the curve separates from the ineffective drug(s)-dose(s) combinations.

RESULTS

Neurite Elongation

Single Growth Factor—FK506 was observed to induce neurite elongation in the lower concentration range, and the average neurite length of DRGs exposed to 1 (633.1 ± 65.3 μm), 5 (682.0 ± 61.0 μm), and 10 (751.3 ± 38.0 μm) ng/ml had statistically longer neurite extensions than the control group (0 ng/ml; 469.7 ± 22.1 μm; $P < 0.05$) [Figs. (1 and 2)a]. In contrast, there was no difference in neurite length between DRGs exposed to 20 ng/ml FK506, 50ng/ml FK506, or control. Treatment of the DRGs with NGF concentrations of 0.1 (910.5 ± 39.8 μm), 1 (993.7 ± 53.6 μm), 5 (1063.5 ± 57.8 μm), 10 (1117.6 ± 55.6 μm) ng/ml, and 100 ng/ml (755.7 ± 57.8 μm) enhanced neurite elongation compared with control (469.7 ± 22.1 μm; $P < 0.05$) (Fig. 2b).

Combination Treatment—The combined response of 2 drugs was compared with control and with their individual doses to determine the response. The response was found to be potentiating when the combination treatment resulted in a better response than what was obtained individually. Certain treatment responses were found to be competitive when the combined dosage resulted in reduction of growth compared with the individual response. If

there was no significant change in growth with the combination treatment when compared with single treatments, the response was listed as noncompetitive.

The combined treatment of FK506 and NGF showed increased neurite elongation compared with their corresponding individual doses (Fig. 3b and 3c). The combination of NGF with FK506 treatment was potentiating and showed increasing neurite elongation with increasing NGF concentration except when NGF was combined with 1 ng/ml FK506, which was competitive (Fig. 4a). Higher FK506 concentrations (20–100 ng/ml), while inhibitory on their own, exhibited significantly increased elongation in combination with NGF and GDNF (seen in shaded region of Fig. 4a). The best combinations were found to be 1 ng/ml NGF with 5– 10 ng/ml FK506, 100 ng/ml NGF with 50 ng/ml FK506, and 10 ng/ml NGF with 20 ng/ml FK506 ($P < 0.05$).

The combined treatment of FK506 and GDNF showed significant increased neurite elongation (shaded region of Figure 4b) as compared with their individual treatments (Figs. 2a,b). The combined treatment of FK506 and GDNF was potentiating for combinations of 1–10 ng/ml of GDNF with 1–10 ng/ml of FK506 (Fig. 3d,e and 5c), and for combination of GDNF 0.1 ng/ml with FK506 50 ng/ml ($P < 0.05$). All other combinations showed a noncompetitive response ($P > 0.05$).

Combined GDNF and NGF had a potentiating effect and produced significant enhancement of neurite outgrowth as compared with individual treatments, shown in shaded regions of Figure 4c. The plots suggest a strong effect of increasing NGF concentration (0.1, 1, and 10 ng/ml). The average neurite length at the optimal GDNF+NGF concentrations (1 ng/ml NGF combined with 1 or 0.1 ng/ml GDNF) ($P < 0.05$) was 1,000–1,100 μm, which is higher than the 800–950 μm neurite elongation observed with NGF or GDNF alone (Fig. 3i and 5e). Altogether, the results indicate a strong interaction of both growth factors. Statistically, combinations of 1 ng/ml NGF with either 0.1 ng/ml, 1 ng/ml, or 10 ng/ml of GDNF were found to produce the best neurite growth response ($P < 0.05$ when compared with control).

Combination treatment with FK506, NGF, and GDNF produced both potentiating and competitive responses on neurite elongation (Table 1). This triple combination group mediated the greatest neurite elongation when compared with all other treatments with 1 ng/ml of each NGF, GDNF, and FK506 enhancing growth to the greatest extent (1282.4 ± 25.1 μm) ($P < 0.05$). All combinations had a potentiating response except for the combinations with 100 ng/ml of NGF and 100 ng/ml of GDNF ($P > 0.05$), which showed a competitive response.

Neurite Branching

Single Growth Factors—FK506 did not promote neurite branching, because there was no statistical difference in neurite branching between the different concentrations tested (P 0.05) (Fig. 6a). NGF and GDNF induced significant neurite branching in the concentration range of 0.1–100 ng/ml ($P < 0.05$) (Figs. 6b,c). Finally, the neurite branching of the NGF or GDNF treated DRG was generally approximately 50% above that observed with the FK506 treated DRGs.

Combined Growth Factors—As seen in Figure 7a, the average neurite branching due to combined NGF+FK506 treatment was not dose-dependent, and the optimum neurite branching response was observed at the combinations of 0.1 ng/ml and 100 ng/ml of NGF with 10 ng/ml of FK506 (0.3 ± 0.01–0.33 ± 0.01) ($P < 0.05$) which was the same as seen with NGF alone (0.34–0.35) ($P < 0.05$). There was a noncompetitive interaction for combinations of NGF 0.1–100 ng/ml with 1– 50 ng/ml of FK506 (0.28–0.33).

Generally, GDNF+FK506 treatment (Fig. 7b) produced a competitive response and reduced neurite branching (0.2–0.3) as compared with those obtained with GDNF treatment alone (0.3–0.4), except when 5 ng/ml of FK506 was combined with 100 ng/ml of GDNF (0.32 ± 0.01) ($P < 0.05$), which had a noncompetitive response.

Figure 7c shows that the optimum neurite branching (0.34–0.35) for combined GDNF+NGF treatment was obtained with 1 ng/ml NGF plus 10 or 100 ng/ml GDNF or with 10 ng/ml NGF plus 10 or 100 ng/ml GDNF ($P < 0.05$). Neurite branching measurements were more NGF dose-dependent than GDNF dose-dependent. Neurite branching displayed a competitive interaction at lower GDNF concentrations and a potentiating interaction at GDNF values >1 ng/ml.

Combined treatment of NGF+GDNF+FK506 did not produce enhancement in neurite branching as compared to individual treatments (Table 2). The combinations of NGF/GDNF (1–10 ng/ml) and FK506 (10 ng/ml) resulted in a noncompetitive response (0.3–0.35) ($P < 0.05$) while the combinations involving higher concentrations of NGF and GDNF (100 ng/ml) caused a competitive interaction (0.15–0.22) ($P > 0.05$). The response obtained was not dose-dependent.

Neurite Growth Rate—The rate of neurite growth was measured over 3 days (Fig. 8). The concentrations of 10 ng/ml of FK506, NGF, and GDNF were chosen from our analysis of neurite growth at 72 h, because they consistently enhanced neurite growth to a greater extent than controls. The growth rate of the control group slowed down by day 2, such that there was no significant difference in neurite length between days 2 and 3 ($P > 0.05$) (Fig. 8). There was a close to linear increase in neurite length over the 72-h time frame, when DRGs were exposed to FK506, NGF, or a combination of the 2 (Fig. 8a). However, the combination of FK506 and NGF had a steeper growth rate compared with single treatment exposure, as evidenced by the longer neurites at 72 h (933.2 ± 21.2 μm) compared with the other experimental groups (NGF: 789.5 ± 23.4 μm, GDNF: 723.1 ± 22.4 μm). When DRGs were exposed to GDNF, there was initially a steep increase in neurite growth, but the rate decreased dramatically between days 2 and 3 (Fig. 8b). In contrast, there was a more linear increase in neurite growth when DRGs were exposed to the combination of FK506 and GDNF; this combination had the longest neurites by 72 h 811.3 ± 37.4 μm (Fig. 8b). For the different groups, the overall daily growth rate over 3 days of incubation was approximately 188.1 ± 21.1, 228.6 ± 33.2, 263.2 ± 42.1, 241.0 ± 37.5, 316.2 ± 23.4, 311.1 ± 21.2, 270.4 ± 25.3, and 320.4 ± 30.1 μm/d for the control group, the FK506, NGF, GDNF, NGF+GDNF, NGF+FK506, GDNF+FK506, and NGF+GDNF+FK506 treatment groups, respectively.

Western Blot—Based on our neurite growth data, we tested some of the optimum dosages from the single and combined groups for protein expression using Western blot analysis (Fig. 9). The protein expression levels shown represent the ratio of target protein to the GAPDH internal control. Western blots showed Akt expression of 14.2% by DRGs treated with a combination of 1 ng/ml NGF and 10 ng/ml FK506. This interaction was potentiating ($P < 0.05$) when compared with the individual treatments of 1 ng/ml NGF and 10 ng/ml FK506, which resulted in 7.7% and 5% expression of Akt, respectively. Similar results were seen for pAkt expression by the NGF+FK506 treated DRGs (~12%) as compared with 10% pAkt expression by single treatment of 1ng/ml NGF or ~9% pAkt expression by 10 ng/ml FK506 dose ($P < 0.05$). This result was in agreement with DRG growth readings (Figs. 2a,b, 4a), which showed that combined treatment of NGF+FK506 enhanced neurite growth more than either drug alone.

The optimum GDNF+FK506 (1 ng/ml GDNF+10 ng/ml FK506) combined treatment for neurite elongation had a slightly competitive interaction resulting in AKT expression of ~4%, which was slightly lower than what was observed from a single treatment of 10 ng/ml FK506 (AKT 5%; pAkt 8.5%) or 1 ng/ml GDNF (Akt ~4%; pAkt ~7.7%). This result is similar to what we observed from DRG neurite elongation data for this treatment (Figs. 2a,c, 4b).

The overall response of the optimal combined treatment of NGF+GDNF+FK506 (1 + 1+1 ng/ml) was the highest with Akt expression of ~16% and pAkt expression ~13% when compared with dual combinations or the single treatments ($P < 0.05$). This result was consistent with the neurite growth readings obtained from DRGs (Table 1).

DISCUSSION

The main findings of this study are that: (1) FK506 primarily enhances neurite elongation; (2) NGF and GDNF promote both neurite elongation and neurite branching; (3) neurite outgrowth is dose-dependent, with optimal concentrations falling in the range of 1–10 ng/ml for FK506, NGF, and GDNF; (4) combining FK506, GDNF, and NGF exerts a potentiating effect on neurite growth; (5) significant increases in neurite growth are observed in the first 48 h of culture; and (6) combinations of FK506, GDNF, and NGF increased protein expression of AKT and phosAKT when compared with individual treatments.

NGF and GDNF are important neurotrophins.[40] They have been shown to promote neuronal growth both *in vitro* and *in vivo*[9] and have been studied extensively.[41,42] They are the 2 most commonly used neurotrophins when selecting or designing a delivery system for nerve growth and have been used in numerous neurorestorative studies.[40–43] NGF has been found to be essential for development and phenotypic maintenance of neurons in the peripheral nervous system.[44] NGF has been considered to be a very powerful and selective growth factor for sympathetic and sensory neurons, especially for survival and axonal outgrowth of sensory neurons.[41,45] GDNF has shown pronounced effects on motor axonal regeneration and has been identified as a key factor for promoting axonal elongation.[40,41,46,47] More recent work has shown that GDNF ligands can regulate pain responses by modulating sensitization of sensory neurons to different stimuli.[48–50] GDNF was also shown recently to

elicit different effects on motoneuron axons versus sensory cell bodies.[51] Delivery of GDNF in a cell therapy model has been shown to prolong lifespan and control disease progression in a rat model of amyotrophic lateral sclerosis.[52]

This study analyzed the potential effects of different dosages of FK506 in combination with other growth factors on nerve growth. *In vivo* tests have shown that systemically delivered FK506 has nerve regenerative properties in addition to being an immunosuppressant.[20,29,30] There is, however, insufficient information regarding the optimal local dose of FK506 for nerve regeneration after injury. To improve this knowledge, we used a bio-activity test system based on DRG explants from chicken embryos. We have tested the effects of FK506, GDNF, and NGF, alone and in combination, on neurite elongation (Figs. 1–4), branching (Figs. 6 and 7), growth kinetics (Fig. 8), and protein expression of AKT and pAKT (Fig. 9). Chicken DRG explants were selected for this testing process because of their availability, low cost, and their ganglion structure associated with Schwann cells. Due to their structure, DRG explants more closely mirror the situation of neurite growth *in vivo* than single neurons.[42] Chicken DRG explants are a widely used model for neurite growth. Chicken embryonic DRGs are readily available and have also been shown to exhibit growth similarities with other animal neural systems.[9,37] DRGs have been used for testing effects of neurotrophic factors and drugs in several animal models.[53] Neurite outgrowth and survival studies have been done using DRGs.[53] We expected that the use of DRGs for testing should provide relevant information on bio-activity and the useful dose of selected drugs. FK506, GDNF, or NGF induced readily visible neurite outgrowth when compared with control cells. FK506 and GDNF primarily promoted neurite elongation, whereas NGF induced extensive neurite branching with neurite elongation. We found that 1–10 ng/ml of FK506, NGF, and GDNF showed the highest level of elongation of DRG neurites.

The dramatic neurotrophic action of combined doses of FK506 and other growth factors on DRGs is extraordinary in terms of sensitivity, because as little as 1 ng/ml FK506 combined with 1 ng/ml NGF/GDNF was neurotrophic. The effect of NGF/GDNF in promoting neurite sprouting and elongation has been shown earlier.[9] On a molecular level, NGF is known to stimulate neurite extension in embryonic DRG neurons by binding to tyrosine kinase A (TrkA) receptors and activating intracellular signaling pathways,[54] and GDNF promotes primarily motor neuron survival and neurite growth.[47] The optimal NGF/GDNF dose of 0.1–10 ng/ml for maximum neurite outgrowth we observed is in agreement with previous data.[9]

Although neurite growth did not differ significantly between treatments with 1 and 10 ng/ml of GDNF or NGF, it declined at higher doses (100 ng/ml). This result is in agreement with findings in rats, where high doses of NGF delayed nerve regeneration by retarding GAP43 production in the early phase (7 days) after axotomy.[15] We observed that combinations of FK506 with high doses of NGF produced a potentiating response in neurite elongation, which suggests that FK506 may help to enhance neurite growth even with higher doses of neurotrophic factors. In certain cases of combined doses, such as combinations of 1 ng/ml or 5 ng/ml FK506 with GDNF, we see a competitive interaction which could reflect negative feedback resulting from the interaction of different protein expressions and signaling pathways used by FK506 and the neurotrophic factors.[55]

No difference in the rate of neurite outgrowth was found between FK506, GDNF, and NGF treatment during the first 2 days of treatment, but NGF yielded a higher outgrowth rate than others between days 2 and 3 of the experiment. Interestingly, the combination of FK506 with GDNF and/or NGF enhanced the rate of neurite outgrowth at all time points over the other treatment groups. The potentiating effect of FK506 with GDNF and/or NGF on initial neurite growth rate should be considered when developing new therapeutic drug delivery systems.

The potentiating effect of FK506 with GDNF and/or NGF on both neurite growth and, to a lesser extent, on neurite branching is an interesting observation. NGF and GDNF have been found to activate Akt and pAkt expression in cells in neuroprotection and neuro-regeneration pathways.[41,44] Akt is a serine-threonine protein kinase. Induction of the serine-threonine kinase activity is critical for cell survival, as well as cell proliferation. Akt is also regulated by growth factor and serum factor signaling through PI3 kinase.[32,36] Data suggest that FK506 potentiates neurite outgrowth through the Ras/Raf/MAP kinase signaling pathway downstream of PI3K-Akt.[28] The phosphatidylinositol-3 kinase–Akt (PI3K-Akt) pathway is one of the major growth factor pathways that has been studied and shows that Akt/pAkt expression is associated with accelerated neurite regeneration after axotomy.[56,57]

Also, Akt has been found to be necessary for neurotrophin-stimulated neurite growth.[36,56] With Western blot analysis, we found that Akt/pAkt activation occurs during neurite growth, and the percentage of protein expression can be related to neurite elongation due to different dosage combinations. In this study, we evaluated whether FK506 individually or in combination with other neurotrophic factors would upregulate the protein expression of Akt and phosAkt. We found that combined application of neurotrophic factors with FK506 enhanced co-expression of both receptors. These results strongly suggest that the interaction of FK506 with GDNF and NGF mediates distinct neurite growth enhancement. Activation of the Akt signaling pathway has been associated with increased neurite growth[58] and would explain the increased expression of receptors.

In conclusion, these results indicate that there is a dramatic increase in nerve growth activity when FK506 is combined with NGF and/or GDNF. NGF and FK506 combined treatments showed potentiating responses for certain dose combinations and had significant effects on neurite growth when compared with their individual doses. Combined GDNF and FK506 treatments exhibited a slightly potentiating effect, but to a lesser extent when compared with FK506+NGF. Combined treatments of all 3 drugs were highly potentiating and resulted in extraordinary effects on neurite growth and branching. However, further investigations are required to elucidate the mechanisms that support the enhanced neurite elongation by combined FK506 and GDNF/NGF treatment. These neurotrophic actions have positive therapeutic ramifications. Numerous neurotrophic proteins, such as brain derived neurotrophic factor, GDNF, and NGF, are being evaluated clinically in various disease states. Difficulties in manufacturing of these large proteins and delivery to target sites create serious barriers to therapeutic application. Drugs such as FK506 are readily synthesized and are thermally stable. Both NGF and GDNF are synthesized endogenously in low amounts at the site of nerve injury, and their efficacy may be enhanced with exogenous delivery of FK506.

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Acknowledgments

Abbreviations

BSA	bovine serum albumin
DMEM	Dulbecco's modified eagle medium
DRG	dorsal root ganglion
FBS	fetal bovine serum
FK506	tacrolimus
GDNF	glial cell line derived neurotrophic factor
NGF	nerve growth factor
PBS	phosphate buffered saline

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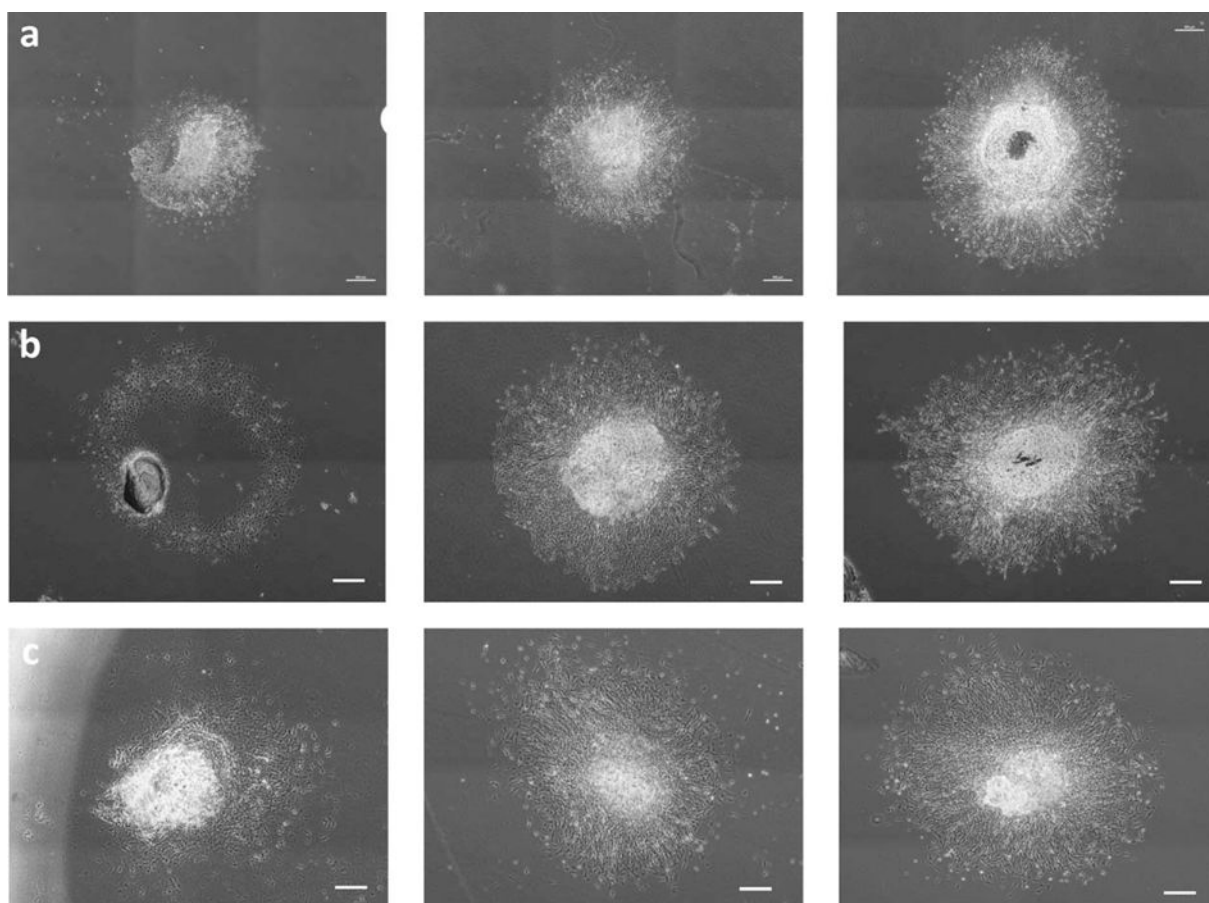
FIGURE 1.
Phase contrast images of DRGs treated with different concentrations of FK506: control (left), 1 ng/ml (center), 10 ng/ml (right) **(a)**; NGF: control (left), 1 ng/ml (center), 10 ng/ml (right) **(b)**; GDNF: control (left), 1 ng/ml (center), 10 ng/ml (right) **(c)**. Neurite outgrowth can be seen based on different doses of FK506, NGF, and GDNF. Scale = 500 μm.
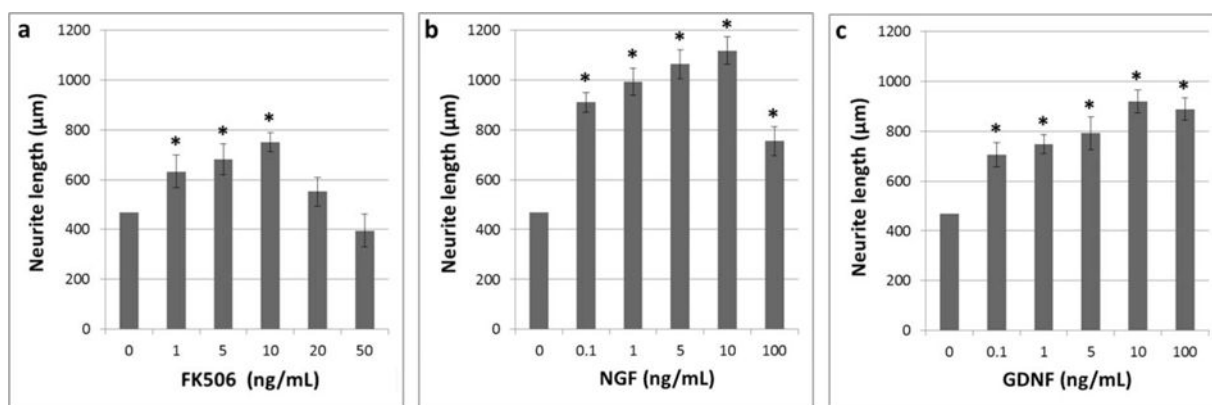
FIGURE 2.
Plots showing effect of single drug treatments on axonal elongation. **(a)** FK506 Dosage/
length. **(b)** NGF Dosage/length. **(c)** GDNF Dosage/length. The bars represent mean ± SD for
$n = 4$ (* statistically different from 0 ng/ml; $P < 0.05$).
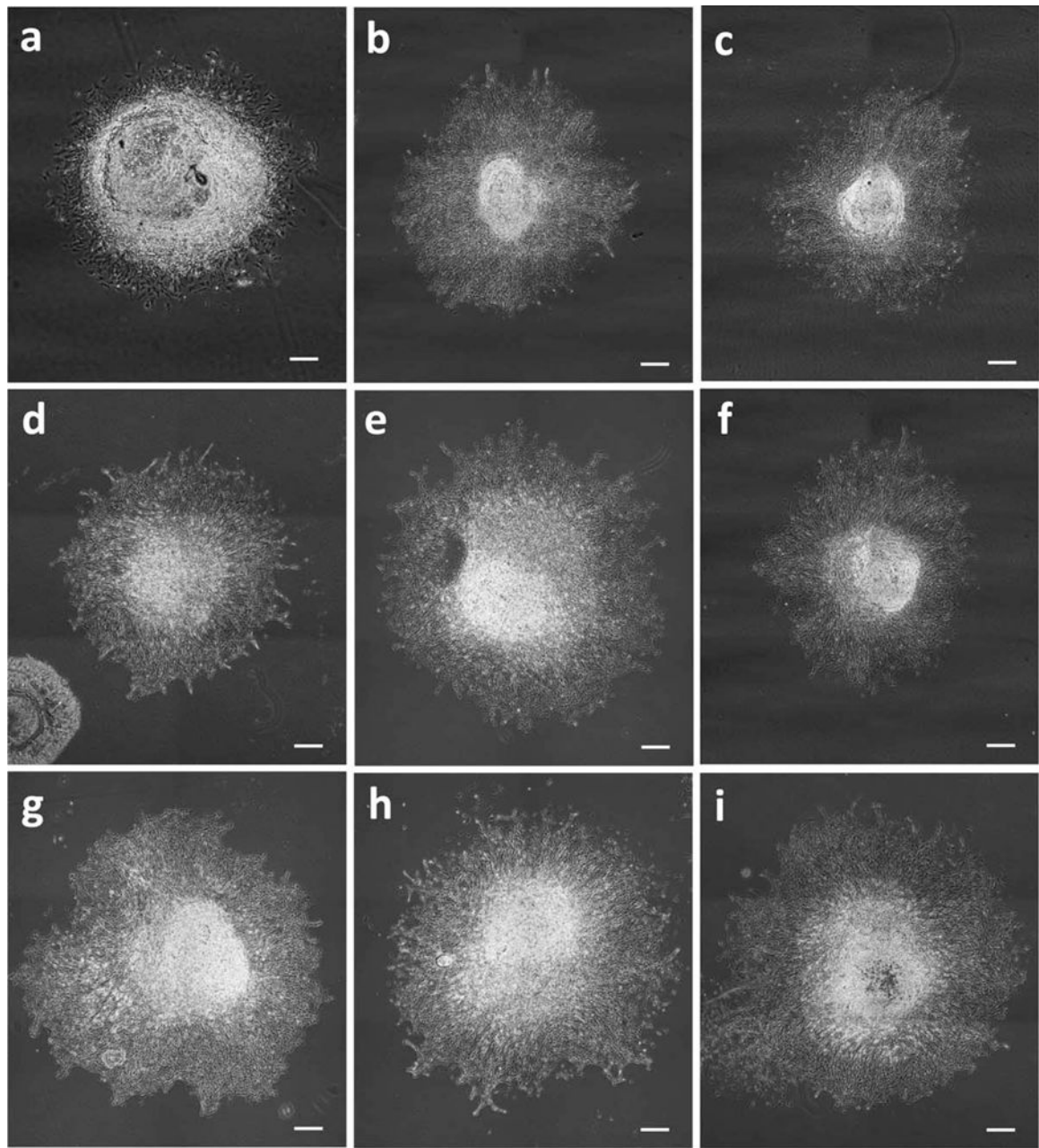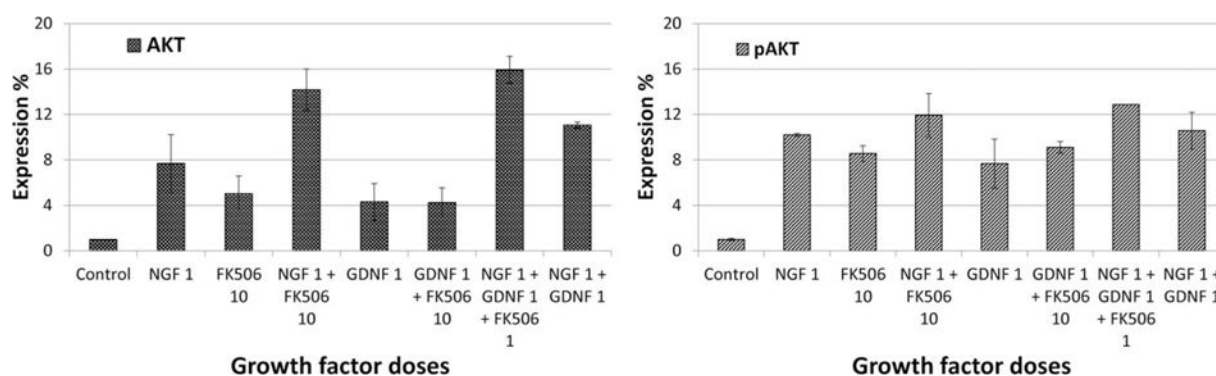
FIGURE 3.
Phase contrast images of DRGs treated with growth factor combinations (starting from top left). **(a)** control (0 ng/ml). **(b)** NGF 1 ng/ml + FK506 1 ng/ml. **(c)** NGF 1 ng/ml + FK506 10 ng/ml. **(d)** GDNF 1 ng/ml + FK506 1 ng/ml. **(e)** GDNF 1 ng/ml + FK506 10 ng/ml. **(f)** NGF 1 ng/ml + GDNF 1 ng/ml + FK506 1 ng/ml. **(g)** NGF 1 ng/ml + GDNF 1 ng/ml + FK506 10 ng/ml. **(h)** NGF 10 ng/ml + GDNF 10 ng/ml + FK506 10 ng/ml. **(i)** NGF 1 ng/ml + GDNF 1 ng/ml. Neurite outgrowth is increased with combined treatment of FK506 with growth factors. Scale = 500 μm.
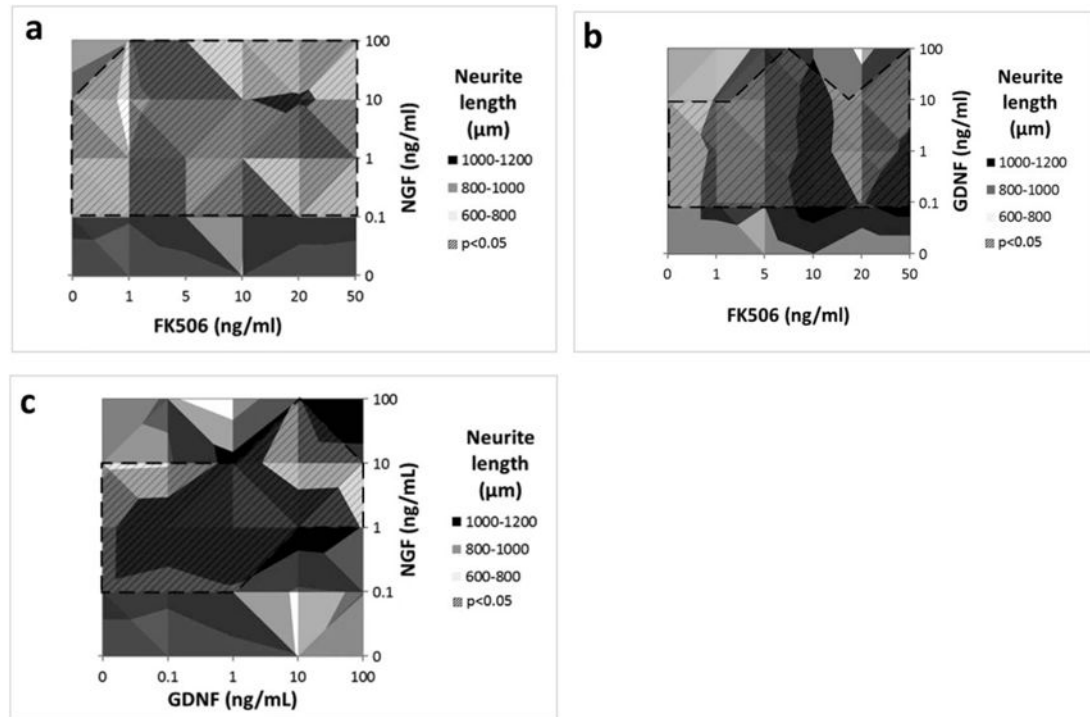
FIGURE 4.
Surface plots showing effect of combined drug treatments on axonal elongation. **(a)** NGF +FK506 combined dosage. **(b)** GDNF+FK506 combined dosage. **(c)** NGF+GDNF combined dosage. (Shaded region with dashed lines specifies the concentration range which produced significantly different axonal elongation response from 0 ng/ml, $P < 0.05$).
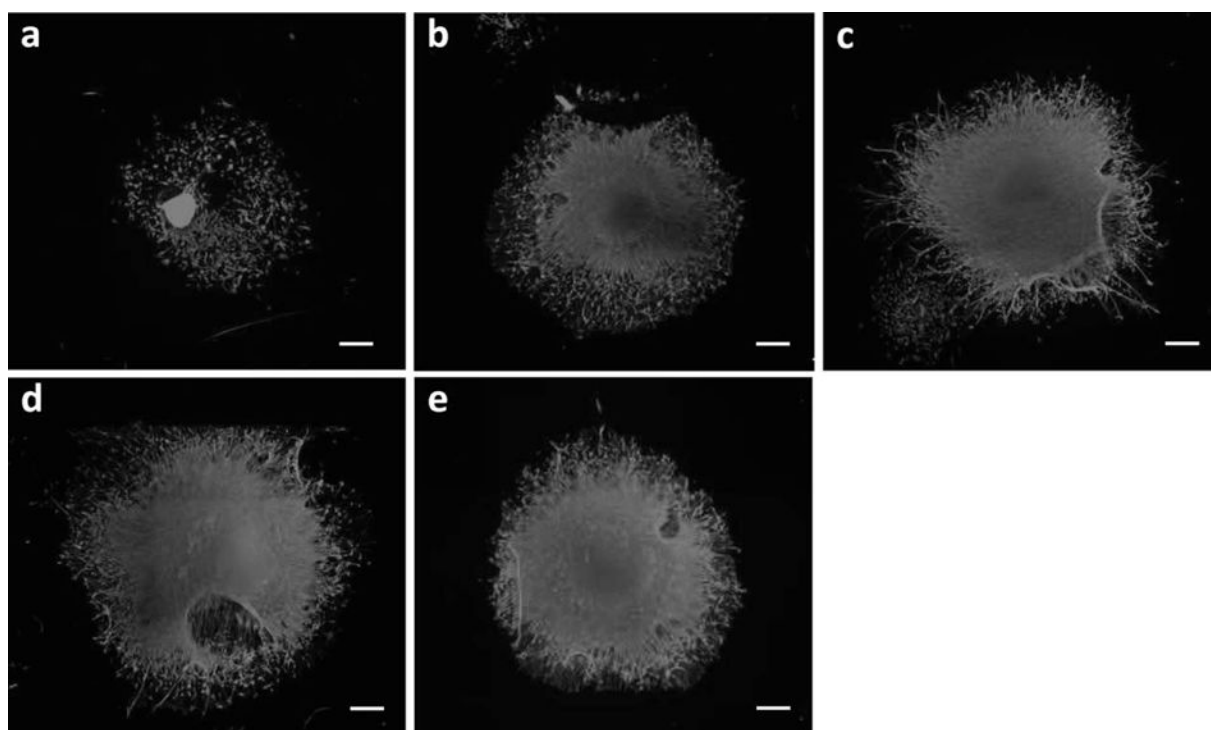
FIGURE 5.
Fluorescence images of DRGs stained with β-tubulin. **(a)** control (0 ng/ml). **(b)** NGF 1 ng/ml + FK506 10 ng/ml. **(c)** GDNF 1 ng/ml + FK506 10 ng/ml. **(d)** NGF 1 ng/ml + GDNF 1 ng/ml + FK506 1 ng/ml. **(e)** NGF 1 ng/ml + GDNF 1 ng/ml. The images show enhanced neurite elongation with the specified treatments.
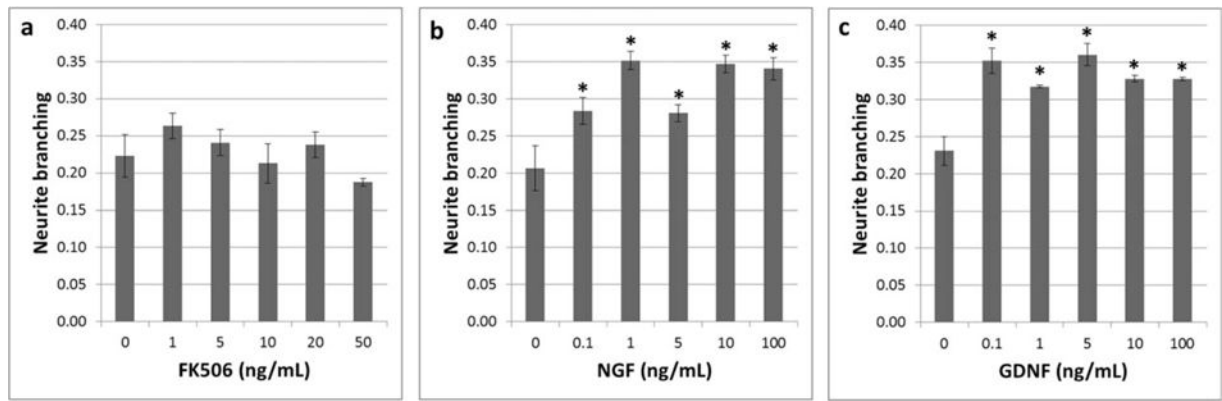
FIGURE 6.
Plots showing the effect of single drug treatment on axon density. **(a)** FK506 Dosage/density. **(b)** NGF dosage/density. **(c)** GDNF dosage/density. The bars represent mean ± SD for $n = 4$ (*statistically different from 0 ng/ml; $P < 0.05$).
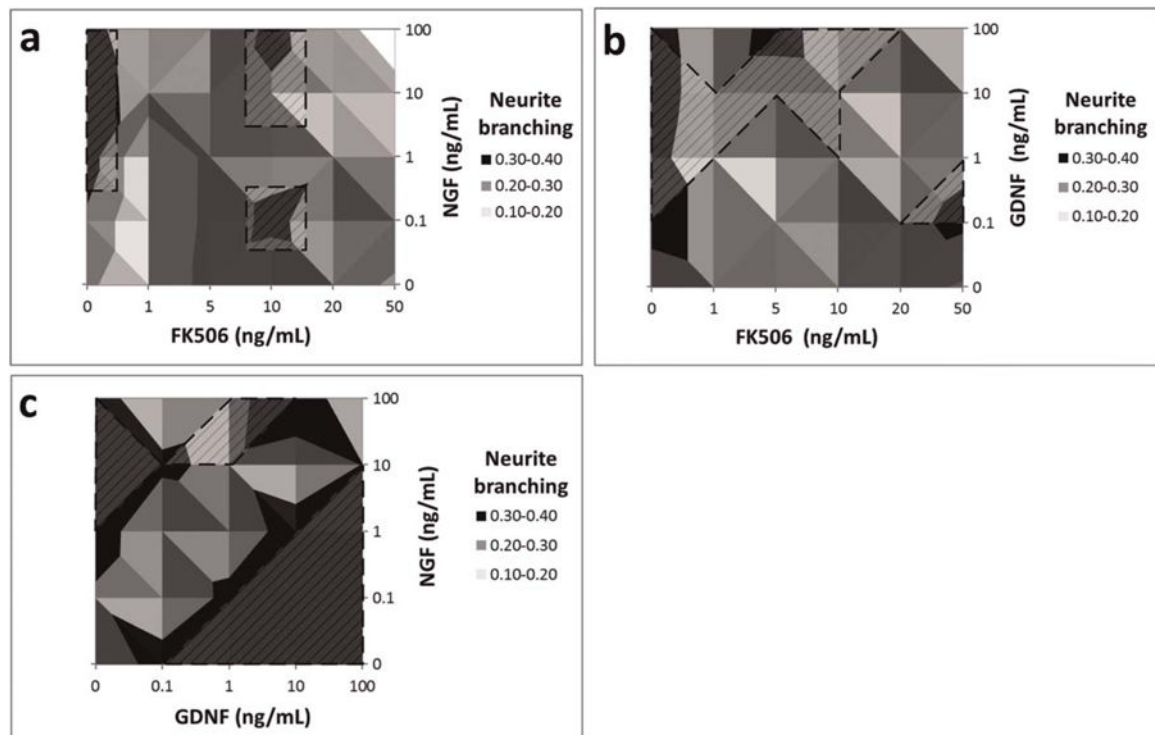
FIGURE 7.
Surface Plots showing the effect of combined drug treatment on axon density. **(a)** NGF +FK506 combined dosage. **(b)** GDNF+FK506 combined dosage. **(c)** NGF+GDNF combined dosage. Shaded region with dashed lines specifies the concentration range that produced significantly different axonal branching responses when compared with 0 ng/ml, $P < 0.05$.
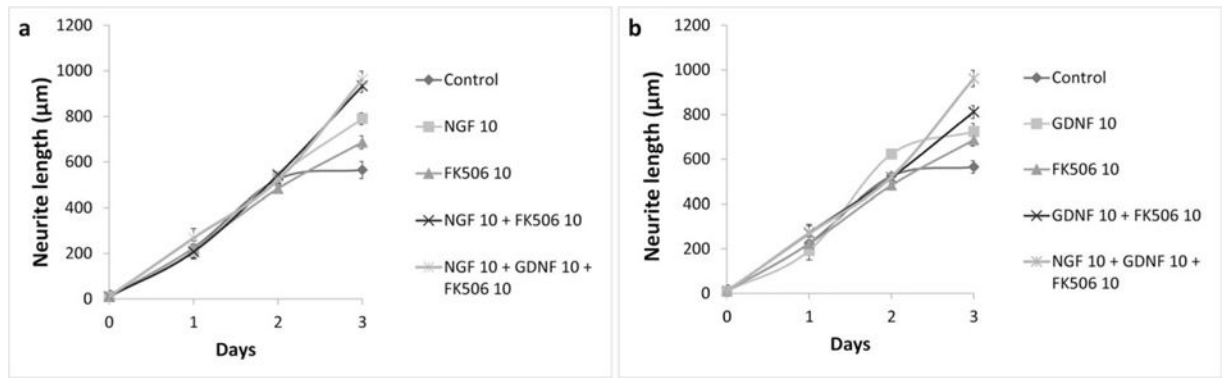
FIGURE 8.

Kinetics of axonal elongation of DRGs. **(a)** treated with FK506 alone (10 ng/ml), NGF alone (10 ng/ml), combined NGF/FK506 (10 ng/ml each), combined NGF/GDNF/FK506 (10 ng/ml each), and untreated (control). **(b)** treated with GDNF alone (10 ng/ml), FK506 alone (10 ng/ml), combined GDNF/FK506 (10 ng/ml each), combined NGF/GDNF/FK506 (10 ng/ml each), and untreated (control). Bars = mean ± SD of $n = 3$. All treatment groups were significantly different ($P < 0.05$) from control (not indicated with asterisks).

FIGURE 9.
Plots showing protein expression of DRG lysates treated with different drug treatments. The bars indicate mean ± SD for $n = 3$ obtained from densitometry of western blot scans.

Table 1

Effect of NGF+GDNF+FK506 combined dosage on neurite elongation.[*]

FK506 (ng/ml)	NGF (ng/ml)	GDNF (ng/ml)	Neurite length (μm)
0	0	0	417.4
1	1	1	1282.4
1	5	10	834.1
1	10	5	765.3
5	5	100	966.6
5	10	1	889.1
5	100	5	781.6
10	1	1	905.7
10	5	1	843.8
10	10	10	1025.7
10	10	10	1036.1
20	1	10	1005.7
20	100	100	743.8

[*] All of the combinations listed were significantly different from 0 ng/ml, $P < 0.05$.

Table 2

Effect of NGF+GDNF+FK506 combined dosage on neurite branching

FK506 (ng/ml)	NGF (ng/ml)	GDNF (ng/ml)	Neurite branching
0	0	0	0.21
1	1	1	0.35
1	5	10	0.36
1	10	5	0.30
5	5	100	0.33
5	10	1	0.33
5	100	100	0.25
5	100	5	0.31
10	1	1	0.16
10	5	1	0.34
10	10	10	0.33
10	10	10	0.30
20	1	10	0.35

PAPER

Novel drug delivering conduit for peripheral nerve regeneration

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Novel drug delivering conduit for peripheral nerve regeneration

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Abstract

Objective. This paper describes the design of a novel drug delivery apparatus integrated with a poly lactic-co-glycolic acid (PLGA) based nerve guide conduit for controlled local delivery of nerve growth factor (NGF) and application in peripheral nerve gap injury. *Approach.* An NGF dosage curve was acquired to determine the minimum *in vitro* concentration for optimal neurite outgrowth of dorsal root ganglion (DRG) cells; PLGA based drug delivery devices were then designed and tested *in vitro* and *in vivo* across 15 mm rat sciatic nerve gap injury model. *Main results.* The drug delivery nerve guide was able to release NGF for 28 d at concentrations $(0.1–10 \, ng \, ml^{-1})$ that were shown to enhance DRG neurite growth. Furthermore, the released NGF was bioactive and able to enhance DRG neurite growth. Following these tests, optimized NGF-releasing nerve conduits were implanted across 15 mm sciatic nerve gaps in a rat model, where they demonstrated significant myelination and muscle innervation *in vivo* as compared to empty nerve conduits ($p < 0.05$). This drug delivery nerve guide can release NGF for extended periods of time and enhance axon growth *in vitro* and *in vivo* and has the potential to improve nerve regeneration following a peripheral nerve injury. *Significance.* This integrated drug delivering nerve guide simplifies the design process and provides increased versatility for releasing a variety of different growth factors. This innovative device has the potential for broad applicability and allows for easier customization to change the type of drugs and dosage of individual drugs without devising a completely new biomaterial–drug conjugate each time.

Keywords: peripheral nerve injury, nerve conduit, drug delivery, neurotrophic factors, reservoir

(Some figures may appear in colour only in the online journal)

Introduction

Traumatic incidents such as vehicle accidents, crush injuries, or lacerations are the primary cause of peripheral nerve injuries, resulting in impaired muscle movement and loss of sensation due to denervation of adjacent tissue and muscles. Peripheral nerve injuries frequently result in paralysis, chronic pain and neuropathies leading to severe disability in patients [1]. Nerve autograft has been the gold standard of treatment for such injuries [2]. This involves the harvesting of donor nerve tissue and implanting the graft across the nerve gap [3]. Nerve autografts have been successful but there have been problems associated with donor tissue availability [3], obtaining a donor nerve via single or multiple surgeries, multiple scarring sites, neuroma formation and the limited success at repairing gaps larger than 10 mm [1]. These issues led to the fabrication of hollow tube-like nerve guiding conduits to span the gap and provide structural support to the transected nerve [4]. There are several FDA approved nerve conduits

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fabricated with collagen (NeuroFlex©, NeuroGen© and NeuroMatrix©), polyglycolic acid (PGA) (NeuroTube©) and poly lactide-co-caprolactone (PCL) (NeuroLac©) [5]. These nerve guides, though structurally helpful, do not effectively replicate a nerve autograft and are not as effective at repairing large nerve gaps [1].

Following a peripheral nerve injury, axons will tend to regrow and cross short gaps, but they need guidance and the ability to overcome the scarring process [4]. To improve the repair process, in addition to providing guidance, therapeutic agents need to be provided at the site of transection or injury [6]. Several drugs and growth factors have been shown to be effective in enhancing axonal outgrowth across nerve gaps [6]. A study of nerve growth factor (NGF) loaded microspheres has shown potential in repairing nerve gaps [7]. Collagen tubes loaded with NGF have demonstrated axonal elongation in *in vitro* studies [8]. Use of NGF within a certain concentration range has resulted in significant neurite growth in chick dorsal root ganglia (DRG) [9]. Several papers suggest that improved peripheral nerve regeneration can be expected by using a nerve bridge and by supplying NGF [6, 10]. To this end, the idea of using a conduit based drug delivery device for nerve regeneration shows great promise since it can both guide and promote axon extension across the nerve gap. The ability of current delivery systems to release adequate quantities of neurotrophic factors (NTFs) to the target tissue is determined by the type of material used to fabricate the delivery system since the materials used may affect the bioactivity of the NTFs, the method of incorporation of NTFs and the mechanism that governs the release of NTFs and other bioactive agents [4]. Drug release from current devices has limitations such as: burst release of NTFs, release dependent upon the degradation of the polymer, and inability to easily switch or add NTFs to the device.

PLGA is a biodegradable material that has shown potential in the development of nerve conduits as a result of its biodegradability, ability to vary degradation time, and biocompatibility [11]. It is a copolymer of poly lactic acid (PLA) and PGA. There have been numerous studies that have demonstrated enhanced nerve regeneration with PLGA nerve conduits [2, 11, 12], especially when used in combination with the release of growth factors [13], the addition of Schwann cells [14], or 3D support structure within the conduit [11]. Though these devices have shown significant results as compared to hollow conduits, they are not easily modifiable for different gap lengths or to deliver different drugs. The use of various drug delivery mechanisms, such as microspheres, coatings, crosslinked polymers or filling with neurotrophin solution in the lumen of conduits, suffers from a lack of flexibility in choosing various drugs and drug concentrations when used with different types of peripheral nerve repair surgery. Thus, a device that is easily customizable in both dimension and drugs (growth factors) of interest would provide a unique solution to the repair of nerve injuries.

Our device is composed of two concentric biodegradable PLGA tubes enclosing a drug reservoir. The reservoir is filled with drug that can access the lumen of the inner conduit through a hole, as shown in figure 1. The expected benefit of the drug reservoir is that a single device design can be used to deliver a variety of drugs to the lumen of the nerve conduit without needing to reengineer the entire system for each drug of interest. The delivery rate of the device should be predictable for any drug, as drug diffusion through an area is well understood and calculable [15], unlike microsphere-based drug delivery systems, which depend on polymer degradation rates. The contents of the reservoir are separated from the lumen of the conduit via a laser drilled hole. This innovative design will allow for controlled release of drug into the lumen of the conduit and potentially enhance growth of the regenerating nerve. The diffusion of each new drug can be controlled with changes in the hole size and reservoir size for controlled drug release. This paper will study the *in vitro* release kinetics of NGF from our device, *in vitro* bioactivity of the released NGF, and *in vivo* efficacy evaluation of the NGF nerve conduit in a rat sciatic gap model.

Methods

DRG explant culture

Fertilized chicken eggs (Merrills Poultry, ID) were incubated at ~39 °C under 100% relative humidity for 12 d. The eggs were first cleaned with 70% ethanol and then opened to collect the embryos. Dorsal root ganglions were dissected from the embryos under a stereomicroscope using a standard dissection procedure [16]. DRGs were separated carefully from connective tissue for culturing in 24-well plates coated with laminin (1 μg ml^{-1}). DRGs were plated at a density of one per well. DRG cultures were maintained in a humid atmosphere at 37 °C and 5% CO_2 for 72 h. Unless specified otherwise, all reagents for cell culture were purchased from Fisher Scientific (Pittsburgh, PA).

Quantification of neurite outgrowth

After 72 h in culture, DRGs were fixed in methanol, imaged using a widefield microscope (Nikon Spinning disk) with a phase contrast lens, images captured with a digital camera at 4× magnification, and average neurite length measured. Neurite elongation measurements were done using a previously discussed procedure [9]. Briefly, the area of the ganglion body (A_{DRG}) and the total area of the DRG with the growing neurites (A_{tot}) were measured using ImageJ 1.31v software (National Institutes of Health, Bethesda, USA). The average neurite length (l_{avg}) was calculated by: $l_{avg} = (A_{tot}/\pi)^{1/2} - (A_{DRG}/\pi)^{1/2}$ [9].

NGF dosing

An NGF dosage curve was obtained to determine the optimal *in vitro* NGF concentration range for neurite growth. Four DRGs of similar size were used for each experiment and the experiments were repeated 3–4 times. Different sets of DRGs were used for each repeat. The DRGs were treated with increasing concentrations of rat β-NGF (R&D systems) (0.1, 1, 5, 10, 50, 100 ng ml^{-1}). For control, DRG-explants
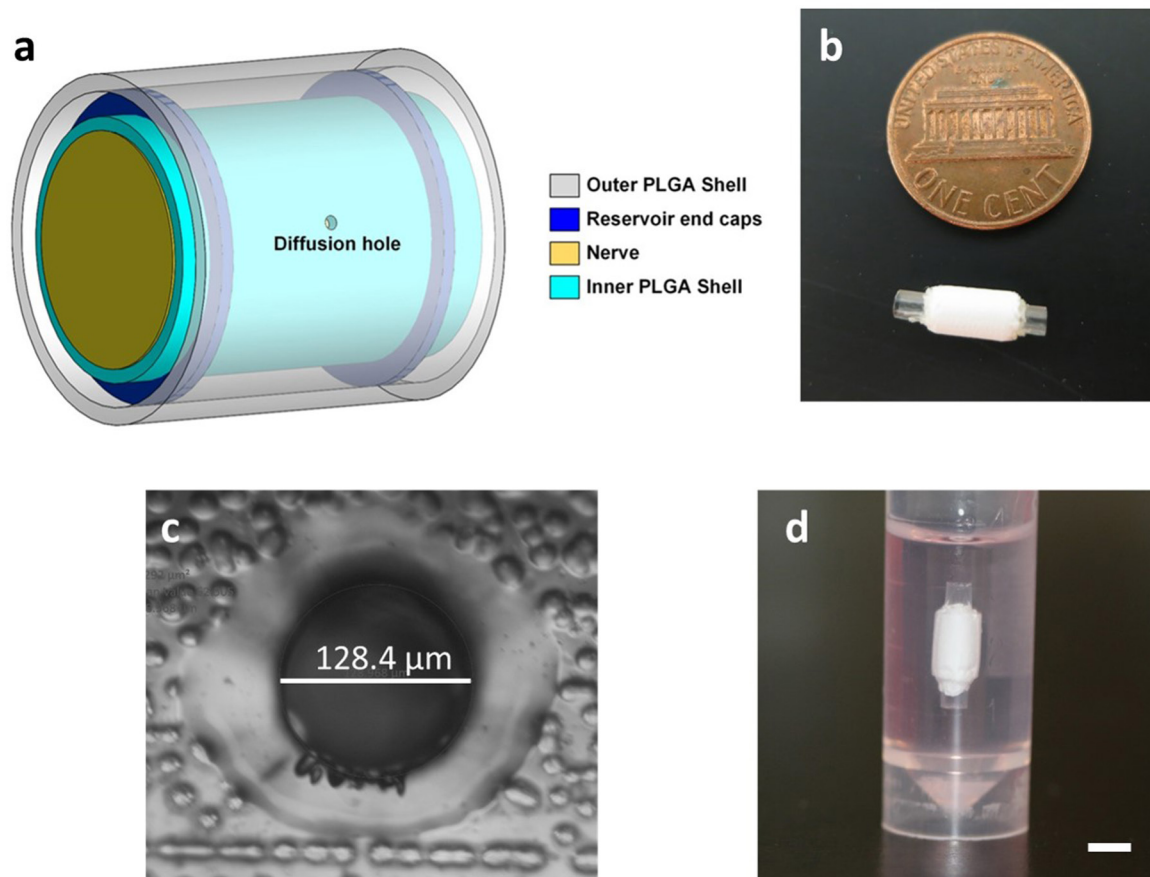
Figure 1. (a) Schematic of the drug delivery device demonstrating structural characteristics including: inner (light blue) and outer (grey) PLGA conduit, drug reservoir (space between the conduits), and end caps defining boundary of reservoir (dark blue). (b) PLGA device with inner and outer tube, as well as a diffusion hole on the inner conduit that enables the release of NGF from the reservoir into the inner chamber. (c) Image of a ~130 μm laser drilled hole in the inner conduit. (d) Device setup in the receiver chamber (scale: 5 mm).

were incubated in cell culture media matrix (DMEM + 10% FBS + 1% Antimycotic) without any growth factor.

Device design

Figure 1 shows the schematic diagram of the proposed device. The device consists of two concentric tubes and the space between the tubes forms the reservoir that stores the drug. The drug is then released into the lumen, with diffusion controlled by the size of the hole made in the inner conduit. The individual device components were manufactured using a solvent casting method as previously described [17]. Diffusion hole of ~130 μm was drilled into the inner conduit using laser milling with a VLS3.60 CO_2 system (Universal Laser Systems). The hole was stabilized by pulsing the laser for multiple passes. This process annealed the PLGA surrounding the diffusion hole and made it more stable once submerged into water. Hole characterization was performed over 28 d to test the effect of PLGA degradation on hole size. ~130 μm sized hole was made in the inner conduit of the device ($n = 5$) using the laser. Multiple passes with the laser were done to anneal the hole boundary and to stabilize the hole. The hole size was imaged using EVOS XL optical microscope and measured using ImageJ over 28 d. A solvent bonding process was adopted to assemble and seal the PLGA conduits as previously explained

[17]. The assembled devices were sterilized using Sterrad 100S system, chosen due to minimal degradation and deformation and verified sterility [17]. Following sterilization, NGF was loaded by injecting through the outer conduit, the injection hole was sealed using a mixture of PLGA and acetone.

NGF-loaded conduit release test

An NGF release test was conducted in a simulated *in vivo* environment as previously described [17]. The conduits used to perform this test were designed according to the size of a rat's sciatic nerve. The drug reservoirs held approximately 20 μl of drug and were loaded with an initial concentration of 0.05 mg ml^{-1} NGF. The devices were sterilized through the Sterrad 100S process and device loading and sealing was done in a bio-sterile hood. The test was conducted using six devices: four devices with 1×130 μm diameter diffusion hole and loaded with an NGF concentration of 0.05 mg ml^{-1} and two sealed devices loaded with 0.05 mg ml^{-1} of NGF with no diffusion hole. Sink method was used for drug collection. Once the fluid was collected, the receiver chamber was flushed and new medium was added to the chamber. The receiver chamber media consisted of DMEM growth media (Thermo Fisher, PA), 10% FBS (Thermo Fisher, PA) and 1% Antimycotic solution (Thermo Fisher, PA). The collected media was used for

evaluation of NGF and bioactivity. NGF concentration in the release media was determine with an enzyme-linked immunosorbent assay (ELISA) (Abcam). A release percentage was calculated by dividing the known released drug mass by the total drug loaded into each device. These tests were conducted at ~37 °C in a 5% CO_2 incubator under sterile conditions.

Bioactivity test

Drug released from the PLGA devices was used to verify and compare the bioactivity of NGF during the 28 d period of drug release. Release eluates from the devices were used for DRG explant culture. Four DRGs were tested per release sample and the test was repeated two times. DRGs were plated at a density of one per well and maintained in a humid atmosphere at 37 °C and 5% CO_2 for 72 h.

In vivo testing

Adult male Sprague Dawley rats ($n = 16$/group) weighing between 150–200 g were used for this experiment as per IACUC approval. *In vivo* efficacy was compared between an NGF delivering conduit and no drug (media filled) conduit (control group) at 21 and 180 d. NGF delivering devices were loaded with ~20 μl of 500 ng ml^{-1} NGF in the drug reservoir. This loading concentration was scaled down and decided based on the *in vitro* release tests so as to obtain NGF release of 0.1–10 ng/ml/d inside the conduit.

Surgery

The rats were anesthetized with isoflurane. The surgical area on the right hind limb was shaved and prepared with alcohol and betadine. A longitudinal (approximately 5 cm) incision was made in the posterior distal thigh of the hind limb, separating the natural muscle planes. Under the operative microscope, a 3 cm segment of the sciatic nerve was isolated. The sciatic nerve was transected to produce a 15 mm nerve gap. The device was sutured to the proximal and distal stumps of the sciatic nerve using a 9-0 nylon suture from the epineurium to the device maintaining the desired gap length. Half of the animals ($n = 8$/group) were sacrificed at 21 d and half of the animals ($n = 8$/group) were left to recover for 180 d.

Nerve histomorphometry

At necropsy, the sciatic nerve including the nerve conduit was harvested, fixed in formalin, and stored in 2% glycine prior to embedding. At the time of embedding, the nerves were post-fixed in osmium tetroxide (2%) for 2 h, dehydrated, paraffin embedded, sectioned, and then stained with hematoxylin and eosin [18]. The sectioned nerve was imaged using light microscopy and analyzed. Three nerve cross sections both proximal and distal to the device were evaluated for number of myelinated fibers, % myelination and *G*-ratio following standard stereological techniques [19]. The degree of nerve growth through the conduit was evaluated by calculating

number of myelinated fibers from light microscope images of the nerve cross sections every 800 μm through the device. Percent myelination was calculated from the distal nerve sections. Distal nerve sections (~10 μm) stained with Osmium tetroxide and Hematoxylin and Eosin were imaged using EVOS XL optical microscope. Thresholding function in ImageJ was used to calculate the percentage of the nerve section area that was myelinated (seen as black due to osmium tetroxide staining). This was calculated to determine the amount of myelination at the distal end of the regenerated nerve.

Motor endplate evaluation

At harvest, the animals were perfused first with heparinized saline and then with 2% paraformaldehyde solution. Following whole body perfusion, the soleus muscle was harvested, fixed in 2% paraformaldehyde for 2 h, cryoprotected in sucrose, embedded in optimum-cutting-temperature compound, and then frozen sectioned ($\approx$25 μm). The frozen sections were used to analyze neuromuscular junction connectivity and density. The sections were stained with alpha-bungarotoxin conjugated to Alexa 594 (1:500; Molecular Probes, OR) to label the acetylcholine receptors and neurofilament (1:25; Biolegend, CA) and synaptic vessel protein (SV2) (1:25 Developmental Studies Hybridoma Bank, IA) to label the nerve [20, 21]. The sections were imaged using confocal microscopy by merging and flattening a stack of images. Multiple randomly identified areas were evaluated for the percentage of reinnervated neuromuscular junctions, again following standard stereological technique [3, 22, 23].

Gastrocnemius muscle

The gastrocnemius muscle of both legs was harvested at necropsy being careful to dissect at the tendinous origin and insertion. The muscles were weighed and the relative muscle mass of the experimental leg was calculated compared with the weight of the contralateral side.

Statistical analysis

The data of *in vivo* tests were statistically analyzed and the differences in experimental groups were compared by a standard *t*-test. Values with $p < 0.05$ were considered significant. The data of NGF growth curve and *in vitro* bioactivity test were statistically analyzed by Anova using Tukey's post-hoc test.

Results

The device was composed of poly lactide-co-glycolide (PLGA) (75:25) to locally release NGF (figure 1). The main device components are as follows: An outer hollow PLGA tube of 3 mm ID and 4 mm OD defines the drug reservoir (figure 1). A second inner PLGA tube of 2 mm ID 2.4 mm OD defines the guidance tube. The space between the inner and outer tubes defines the reservoir volume, which stores
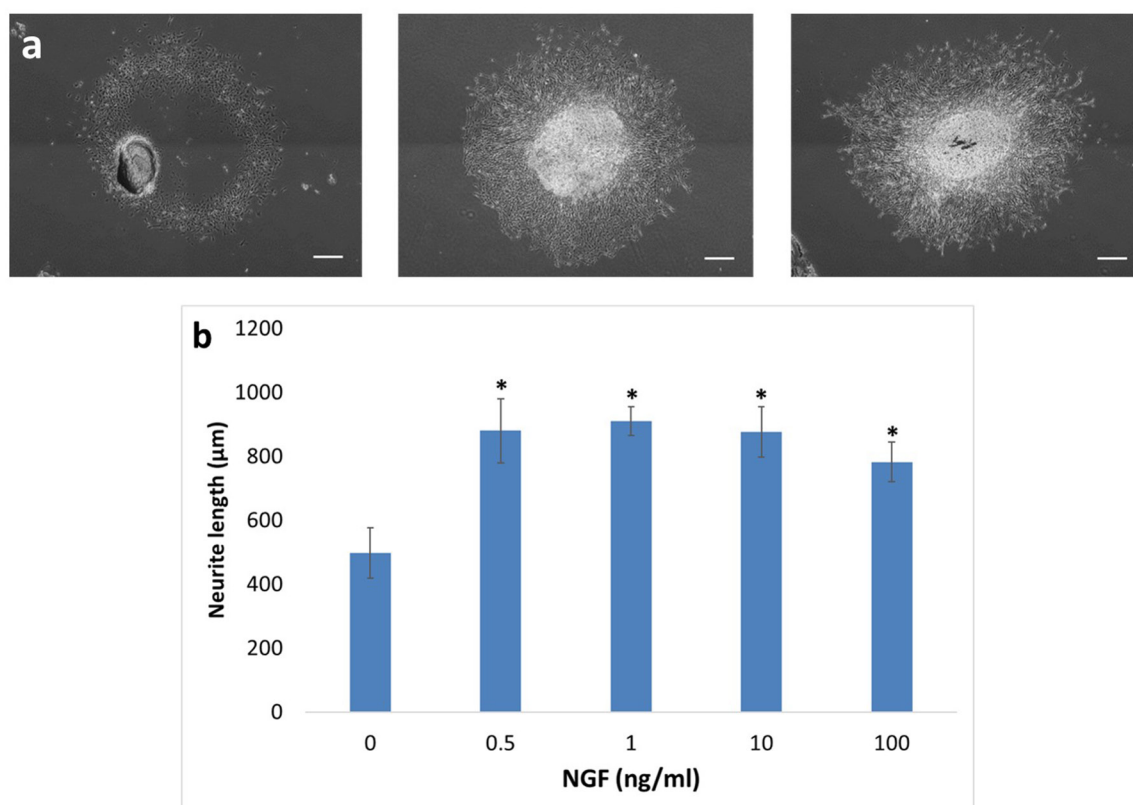
Figure 2. (a) Phase contrast images of DRGs treated with different concentrations of NGF: control 0 ng ml^{-1} (left), 1 ng ml^{-1} (center), 10 ng ml^{-1} (right). (b) Plot showing effect of standard NGF dosages on DRG neurite elongation. Scale bar 500 μm (*indicates statistically different from 0 ng ml^{-1}; $p < 0.05$).

NGF. A laser machined hole of 128 μm diameter (figure 1) was created in the inner tube which defines the diffusion area for drug to transport from the drug reservoir to the conduit lumen. Hole characterization data showed that the device maintained its structure and the hole size varied by <5%.

NGF growth curve

An NGF dosage curve was obtained to determine the *in vitro* concentration required for optimal neurite outgrowth on the DRG cells. Figure 2 shows the images of DRGs and a plot of neurite outgrowth against different NGF dosages. NGF was observed to induce neurite elongation, with DRGs exposed to 0.1–100 ng ml^{-1} NGF having statistically longer neurite extensions than the control group (0 ng ml^{-1}; $p < 0.05$)) (figure 2).

NGF release test

Figure 3 shows local NGF concentration in the receiver chambers for PLGA devices in the 28 d release study. The device design goal was to release NGF at a concentration between 0.1 and 10 ng ml^{-1}, as suggested from the NGF dosage curve results (figure 2). There was a burst release in all devices tested, with $4.5 \pm 1.7\%$ released from the devices in the first 24 h. The data for burst release is not shown on the graph. This burst may be the result of excess drug that was left on
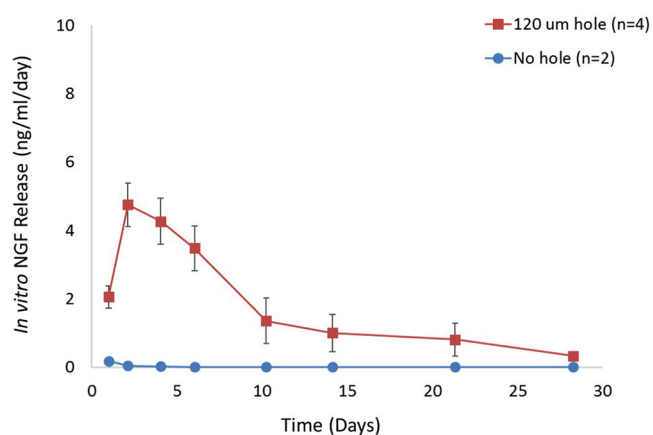

Figure 3. NGF release concentration in the receiver chamber over a period of 28 d *in vitro*. Plot shows average release concentration (ng ml^{-1}) for 1×130 μm hole device loaded with 0.05 mg ml^{-1} of NGF ($n = 4$) (red line); device with no holes loaded with 0.05 mg ml^{-1} of NGF ($n = 2$) (blue line).

the outside of the device when filling or drug quickly passing through the hole during filling or between the fill time and the beginning of the experiment. This initial drug release was then washed away after replacing the media of the receiver chamber several times. Drug releasing devices (0.05 mg ml^{-1} NGF and 1×130 μm hole) released NGF within a range that enhances neurite outgrowth (0.1–10 ng/ml/d) *in vitro*. The release kinetics suggest that loading the conduit with a concentration of 0.05 mg ml^{-1} NGF is able to maintain a
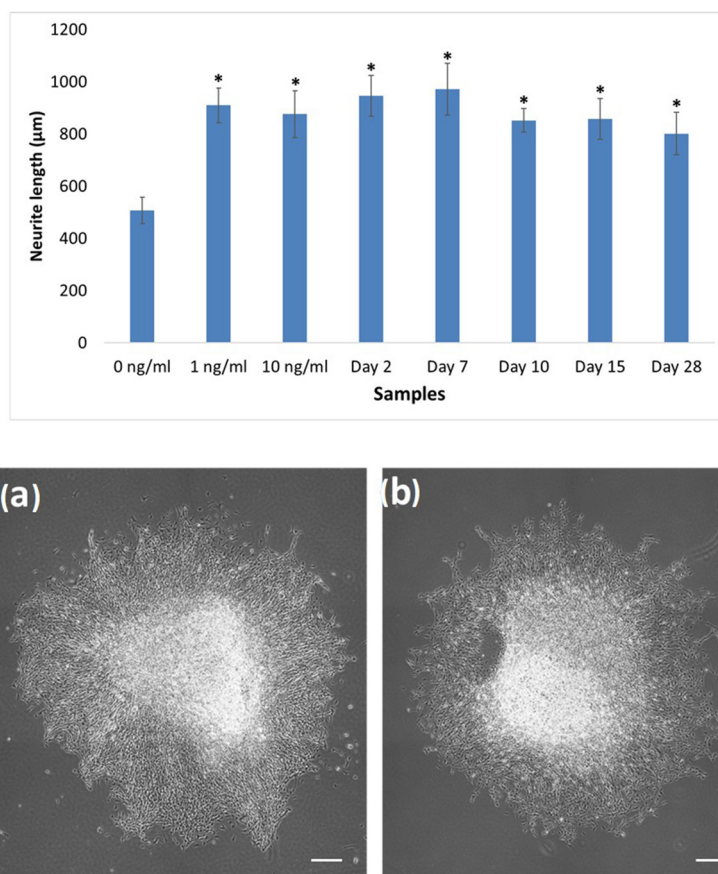
Figure 4. Bioactivity of NGF release collected from the devices. Bar graph shows average neurite elongation (μm) of DRGs treated with NGF released from devices 1 and 2 compared to DRGs treated with freshly prepared NGF (1 ng ml^{-1} and 10 ng ml^{-1}). Scale bar 500 μm (*indicates statistically different from 0 ng ml^{-1}; $p < 0.05$). Phase contrast image of DRGs treated with (a) bioactive drug released from device 1 d 10 release (4 ng ml^{-1}) and (b) bioactive release from device 2 d 10 release (6.8 ng ml^{-1}). Scale bar: 500 μm.

concentration within the therapeutic range for 28 d. The sealed devices (0.05 mg ml^{-1} NGF and no diffusion hole) had minimal leakage in the first 3 d with the average starting concentration of 1 ng ml on day 1 and dropped off to ~0.02 ng ml^{-1} in the later collections, verifying that the reservoir was sealed and adding confidence that the drug collected from the receiver chamber was released through the inner conduit diffusion hole.

Bioactivity

Before moving to *in vivo* testing, it was important to verify if the device was releasing bioactive NGF in a controlled manner at a desired concentration. In order to evaluate the bioactivity of the released NGF we exposed DRG cells to the collected release media and evaluated DRG neurite extension after 72 h. We evaluated media collected on days 2, 7, 10, 15 and 28 from the release test. As a reference, we also exposed DRG cells to varying concentrations of freshly prepared NGF (0, 1 and 10 ng ml^{-1}), as shown in figure 4. The results show that the released NGF retains bioactivity, as DRGs exposed to released or fresh NGF had enhanced growth compared to the control group ($p < 0.05$) and there were no differences between the neurite length between the released media and freshly prepared NGF ($p > 0.05$).
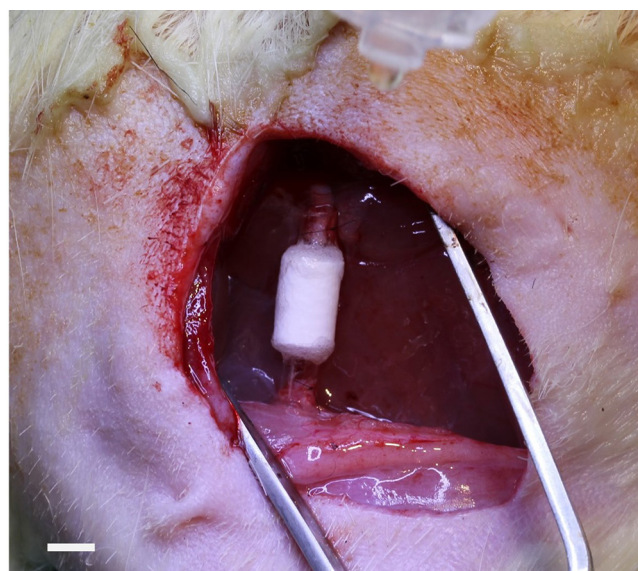

Figure 5. Image of PLGA drug delivery device implant across a 15 mm sciatic nerve gap injury (scale: 5 mm).

In vivo testing

The *in vivo* testing of this device was designed to evaluate nerve regeneration using a rat sciatic nerve injury model. We compared nerve regeneration between animals that had a gap
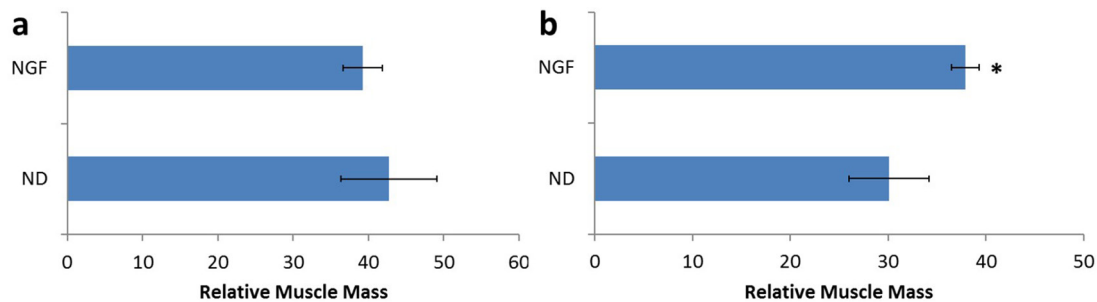
Figure 6. Relative muscle mass ratio (experimental/contralateral) at (a) 21 d and at (b) 180 d. ND is the no drug conduit. * indicates significant statistical difference between the groups at $p < 0.05$.
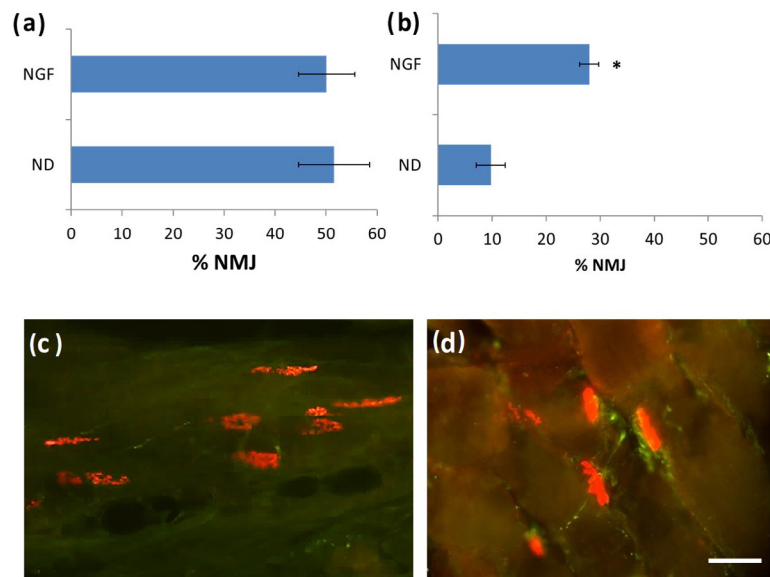

Figure 7. Motor endplate reinnervation (a) % acetylcholine receptors reinnervated at 21 d and at (b) 180 d (c) image of the innervated neuromuscular junction (control group at 180 d) (d) image of the innervated neuromuscular junction (group: NGF conduit group at 180 d). Green stain shows the nerve entering the red stained acetylcholine receptors. Control is the no drug conduit. * indicates significant statistical difference between the groups at $p < 0.05$. NMJ = neuromuscular junction. Scale bar: 30 μm.

injury repaired with a media filled conduit (control group) or a conduit that released NGF (NGF conduit). Macroscopically, all rats showed growth from the proximal to distal end. The main outcome measures were nerve histology, relative muscle mass and motor endplate reinnervation. NGF level in the serum was evaluated at 21 d (3.2 ± 2.9 pg ml^{-1} NGF group; 0.5 ± 0.2 pg ml^{-1} control group) and at 180 d (4.0 ± 1.4 pg ml^{-1} NGF group; 3.6 ± 0.4 pg ml^{-1} control group), with no significant difference between the two experimental groups ($p > 0.05$).

Gastrocnemius atrophy

At 21 d, there were no group differences between the control group and NGF conduit group in terms of gastrocnemius relative muscle mass ratio (figure 6(a)). The gastrocnemius muscle weight at the experimental side was approximately 40% of the contralateral side for both of the experimental groups. This was in contrast with the muscle mass ratio results at 180 d (figure 6(b)) where the NGF conduit group (37.9 ± 1.4%) had significantly higher muscle mass than the no drug group (30.1 ± 4.1%) ($p < 0.05$).

Motor endplate evaluation

At 21 d and 180 d we evaluated the degree of connectivity between acetylcholine receptors and nerve fibers. The data is reported as the percentage of acetylcholine receptors connected to nerve fibers. There were no significant differences in the percentage of acetylcholine receptors connected to nerve fibers between the two groups at 21 d (figure 7). At 180 d, the NGF conduit group had more receptors (28.1 ± 2%) attached to nerve fibers compared with the control group (9.7 ± 1.3%) ($p < 0.05$). The neuromuscular junction data supported the relative muscle mass as shown above.

Nerve histomorphometry

At 21 d, the conduit was present and we observed that the distal nerve histology in both the control group and NGF conduit did not have any myelinated fibers. At 180 d the conduit in both the groups had completely degraded. Regenerated nerves were characterized by multiple fibrous layers surrounding the nerve tissue (figure 8).

There were significant differences between the NGF filled conduit and no drug conduit in terms of % myelination and
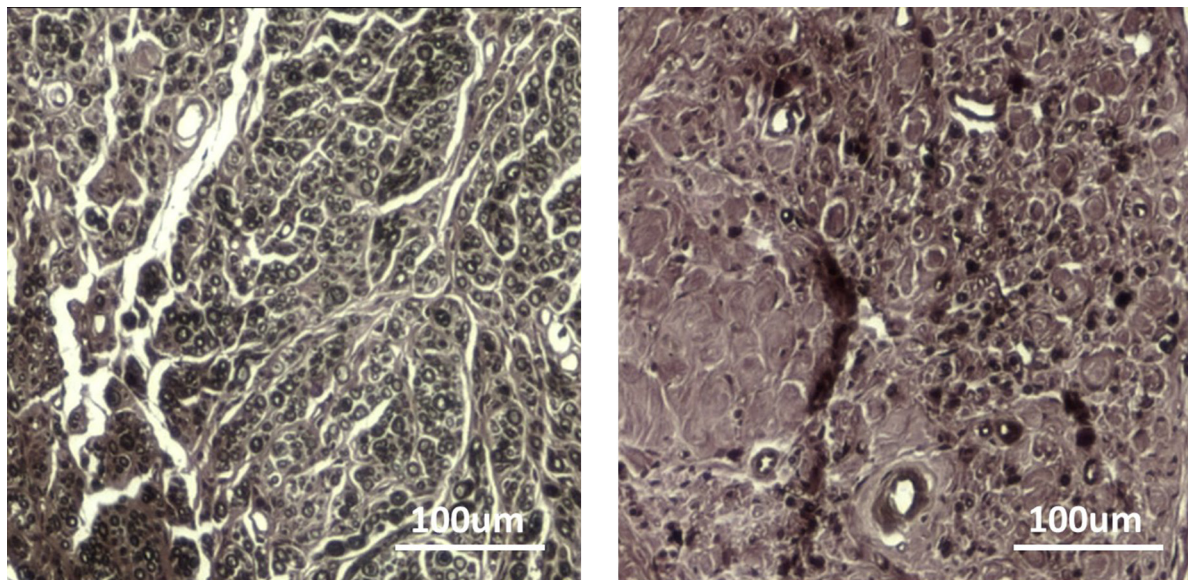
Figure 8. Representative images of the transverse nerve cross section at the distal end (left) group: NGF conduit (right) control group: no drug conduit. NGF treated nerve demonstrates normal morphology with significantly more myelinated axons compared to no drug conduit. Scale bar: 100 μm.

number of myelinated fibers at the distal end with NGF loaded conduits having ~20% more myelination at the distal end than the control (no drug) conduits ($p < 0.05$) (figure 9). Figure 9 shows the number of myelinated fibers across the entire cross section of the conduit for both NGF loaded and no drug devices. The *G*-ratio of both the groups was similar (~0.5), indicating that the maturity and myelination of the regenerated fibers was comparable.

Discussion

Bridging the nerve gap is one of the biggest challenges in reconstructive surgery. Growth factors such as NGF have been shown to enhance axonal outgrowth in several studies [7]. *In vivo* NGF treatment has shown more myelination after nerve gap or crush injury [24, 25]. These studies show beneficial effects in the short term and are dependent on the consistency of drug release over the treatment period. NGF needs to be present longer and within an adequate dose range to sustain regenerative effects over a long period of time. The main outcomes of our study revealed that: (i) a PLGA drug delivery conduit was designed that could release optimum NGF concentrations (0.5–10 ng ml^{-1}) locally (ii) the released NGF was found to be bioactive and could enhance DRG outgrowth and (iii) releasing NGF from our device across a 15 mm sciatic nerve gap improved muscle weight, myelinated nerve growth, and neuromuscular junction connectivity to a greater degree than the control group.

One of the main issues when incorporating drugs into a nerve conduit is the drug availability and half-life. Various materials have been used to build nerve conduits to replace an autograft and for drug delivery [1]. The ability of a conduit based delivery system to release adequate quantities of neurotrophic factors to the target tissue is determined by the type of material used to fabricate the delivery system since the end

degradation product of the materials used may affect the bioactivity of the growth factors, the method of incorporation of neurotrophins and the mechanism that governs the release of neurotrophins and bioactive agents [2]. The delivery of proteins is considered problematic due to their complex nature and stability [1]. The different type of growth factor delivery systems currently being researched are based on degradable polymers [3], affinity interactions [4–7], swelling and erosion of microspheres [8–13], crosslinked polymer [14–16] etc.

Comparing hole based drug delivery systems with commonly cited polymer degradation based drug delivery systems in terms of release kinetics, ease of switching drugs, ease of manufacturing and flexibility of use with different nerve injury treatments, though there have been systems capable of delivering controlled and tunable release kinetics, our device provides the flexibility (in terms of use in different nerve injury scenarios and drug choice) and tunable drug release [17]. Each of the approaches cited above have shown significant benefits when compared to autograft and hollow conduits, but they are not easily modified or easily understood for use in different scenarios such as when using a different drug or treating different length nerve gaps or using across a graft/scaffold. All of these approaches provide factors that encourage the growth of nerves into the conduit, but have accompanying limitations. As an example, when embedding growth factors within a polymer matrix, it is difficult to switch between different growth factors as the release is dependent on the degradation of the polymer and the release varies based on several factors, making prediction difficult. When embedding growth factors in a polymer, the release is dependent on degradation of the polymer which can be affected by temperature, acidity, etc and this type of degradation often results in a high initial release from surface erosion and a decreased delayed release [18, 19]. Given the limitations, there remains the potential to improve peripheral nerve regeneration with a new device.
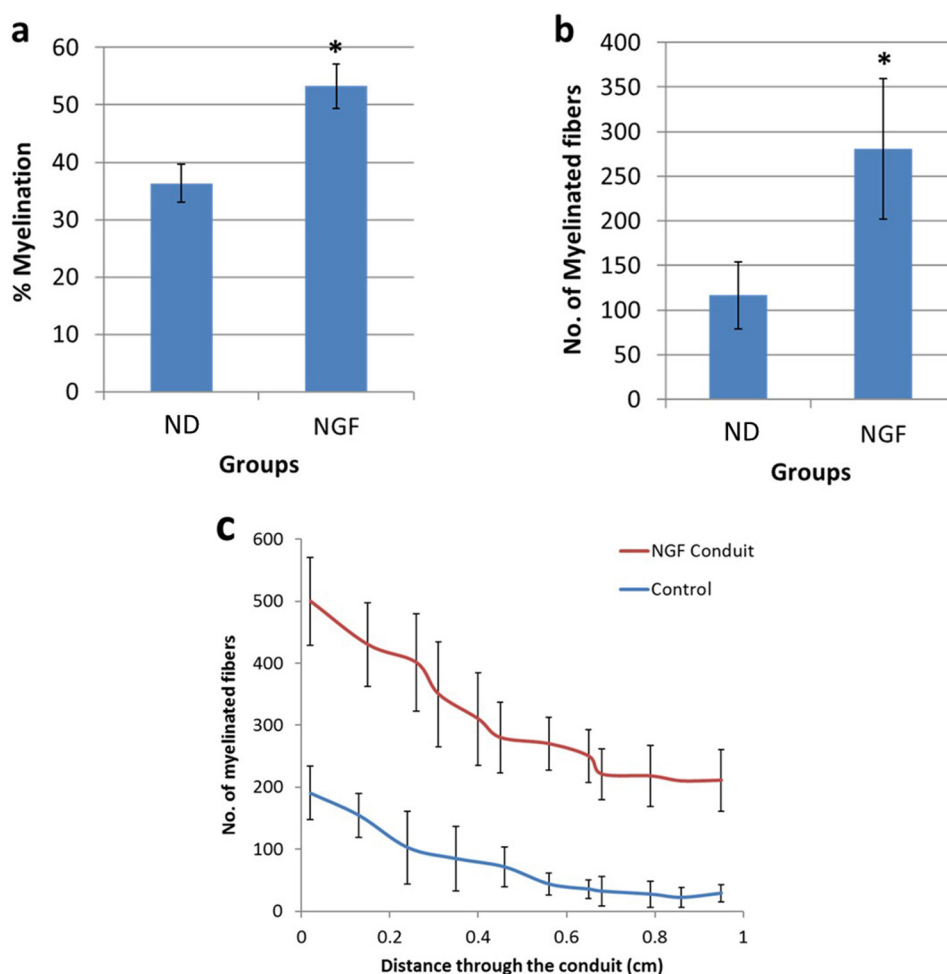
Figure 9. Nerve histomorphometry at 180 d (a) % myelination at the distal end (b) no. of myelinated fibers at the distal end (c) no. of myelinated fibers through the nerve conduit. Control is the no drug conduit. ∗ indicates significant statistical difference between the groups at $p < 0.05$.

Encapsulation of therapeutic molecules within polymers is a well-established method for achieving controlled release, yet issues such as low loading, burst release, poor encapsulation efficiency, and loss of protein activity have limited clinical translation [13, 20, 21]. By taking advantage of the simple physical process of diffusion a hole based drug delivery system was designed that demonstrated controlled release without encapsulation. In fact, we have been able to use this drug delivery device to obtain extended-release profiles for proteins or small molecules without encapsulation [17, 22]. The main components are two concentrically placed conduits with two rings at the ends. The space between the inner and outer tube of the device defines the reservoir volume, which stores the drug of choice. A laser machined hole in the inner tube is created to define the diffusion area for drug to transport from the drug reservoir to the regenerating nerve. The diffusion occurs only across the diffusion hole and not through the outer wall of the tube. This design is advantageous as the amount of drug released is not dependent upon polymer degradation, but upon the loaded drug concentration, diffusion hole size, and drug reservoir volume. This allows for ease in changing the drug of choice (growth factors or small molecules) and ease in manufacturing since the basic device design

can be the same regardless of the chosen drug and the release can readily be predicted. The only variables that need to be manipulated are those that alter drug diffusion (i.e. diffusion hole size, drug concentration or reservoir volume).

Hole characterization was performed to test whether the diffusion holes were changing during the testing procedures (submerged in media matrix and at 37 °C) and dimensional instability may lead to unpredictable diffusion of drug. *In vitro* release data suggests that there was not any significant burst release from the devices and that the drug release was controlled within the range as expected based on the hole size. The devices were designed to provide continuous and controlled NGF release within the therapeutic range over a period of 30 d. The device maintained its structure and the hole size was significantly less. Additionally, the released drug concentration was within our expectation with $\pm 10\%$ variation. Annealing around the hole due to multiple laser passes resulted in most accurate results and highly repeatable holes over 28 d.

Based on the *in vitro* release test, we found that our device had released up to 20% of the total drug loaded in the reservoir through the hole within 30 d. The no hole devices had released significantly less ($<1\%$), showing that the drug

released through the lasered hole and not due to polymer degradation. Compared to the cited studies, concentration of the released drug from our device was less but it was bioactive and therapeutic and gave credibility to the fact that controlled and burst free release could be obtained using a hole based drug delivery system.

The DRGs (primary cell line harvested from chicken embryos) treated with released NGF had significant neurite elongation (+120%) compared to no drug treated DRGs. The rat sciatic nerve gap of 15 mm (critical gap length for a rat) treated with our drug delivery conduit significantly improved gastrocnemius muscle mass in the experimental leg (+30%) and nerve myelination (+100%) compared to no drug treated animals. Comparing our device with the current literature, we have presented an encapsulation free, burst free drug delivering conduit which can preserve drug bioactivity up to 30 d and is significantly therapeutic when used across 15 mm rat sciatic nerve gap injury.

Compared to the literature, a decreased number of myelinated axons were counted in our study. The number reported was significantly higher (>100%) when compared to our no drug control showing that NGF release from our device had significantly improved nerve regeneration across a 15 mm nerve gap. Unfortunately, it is not possible to directly compare the number of axons from another study to our study. A head to head comparison of animals undergoing a controlled study would be required to make a valid comparison.

We were able to evaluate nerve regeneration not only by histomorphometric outcomes, but we have evaluated nerve regeneration based on relative muscle mass and motor endplate reinnervation as well. All outcomes inform us about the different aspects of regeneration. Our study evaluates a novel drug delivering strategy for nerve regeneration. The NGF loaded conduit is compared with a no drug conduit. It shows that the NGF delivering conduit has more myelinated fibers at 180 d than the no drug conduit. Regenerative fibers in the conduits have a longitudinal hourglass shape, with the smallest cross-sectional area in the middle. All results were normalized to gap length as measured by the suture to suture distance at harvest. While the initial suture-to-suture distance was 15 mm in both groups, at harvest, it was noted that in some animals, the suture distance changed, either due to motion and stretching or due to degradation of the conduit.

Enhanced neurite sprouting with NGF treatment as shown in DRGs could explain the higher number of myelinated fibers in the NGF conduit group. The quality of regenerated fibers between the two groups does not seem significantly different. The *G*-ratio was unaffected by the administration of NGF after 180 d of regeneration. When assessing relative muscle mass and neuromuscular junction staining, the groups showed similar results at 21 d. At 180 d, the NGF conduit group had significant differences compared to the control group. NGF has been shown to enhance motor and sensory regeneration [6, 24–26] which could account for these differences. Serum NGF level was evaluated at 21 d and 180 d using an ELISA. There were no significant group differences in terms of serum NGF concentration between the two groups, suggesting that the improved outcomes in the NGF conduit group is due to local delivery of bioactive NGF.

In a previous study, we have shown delivery of bioactive FK506, a small molecule, from a biodegradable PLGA conduit [17]. In this study, we show that our PLGA conduit delivery system delivers bioactive concentrations of a growth factor at a controlled rate and subsequently enhances nerve growth. Our device does not inhibit or obstruct outgrowing axons, when placed across a nerve gap, and is fully resorbable. This is the first requirement for design and use of a delivery system. This drug delivery device only needs to be applied once, during implantation, and does not necessitate surgery for removal. This reduces infection risk, which is present when implanting reservoirs or osmotic pumps.

This innovative device has the potential for broad applicability. In the future, this device could be used to deliver multiple drugs by creating separate drug reservoirs and diffusion areas. These could include growth-factors, small molecules, and synthetic or natural proteins. Our device also has the potential to function synergistically with stem cells or Schwann cell transplants by delivering proteins or growth factors to the cells to direct differentiation or function. This approach allows for easier customization to change the type of drugs and dosage of individual drugs without devising a completely new biomaterial–drug conjugate each time. Our future aim is to evaluate the *in vivo* efficacy of this device compared to an autograft and potentially enhance nerve repair through the local delivery of more than one growth factor or drug.

Conclusion

This study shows that our device can release optimal levels of bioactive NGF at a controlled rate for 28 d. The NGF dosage curve sets the *in vitro* NGF concentration requirement of $0.1–10 \, \text{ng ml}^{-1}$ for optimal neurite outgrowth of DRG cells. PLGA devices loaded with different NGF concentrations were tested, and the devices met the requirement to maintain an optimum *in vitro* release concentration for a period of 28 d. Release samples were tested for bioactivity, and the neurite outgrowth of the DRG cells confirmed that the released drug (NGF) was still active on the 28th day of the release study. Additionally, the *in vivo* testing of the device in a 15 mm rat sciatic nerve defect model demonstrated that locally releasing NGF from our device improved muscle weight, myelinated nerve growth, and neuromuscular junction connectivity to a greater degree than with the device alone (no NGF). The results of this study demonstrate that the incorporation of a novel delivery system providing controlled release of growth factors enhances peripheral nerve regeneration across critical nerve gaps.

Acknowledgments

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Controlled delivery of FK506 to improve nerve regeneration

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Abstract

Autologous nerve grafts are the current "gold standard" for repair of large nerve gaps. However,

they cause morbidity at the donor nerve site, only a limited amount of nerve can be harvested,

and there is the potential for mismatches in size and fascicular patterns between the nerve stumps

and the graft. Nerve conduits are a promising alternative to autografts and can act as guidance

cues for the regenerating axons and allow for tension free bridging, without the need to harvest

donor nerve. Separately, FK506, and FDA approved small molecule, has been shown to enhance

axon growth and peripheral nerve regeneration. This paper describes the design of a novel drug

delivery apparatus integrated with a PLGA (poly lactic-co-glycolic acid) based nerve guide

conduit for controlled local delivery of FK506. An FK506 dosage curve was acquired to

determine the minimum *in vitro* concentration for optimal axonal outgrowth of DRG (Dorsal

root ganglion) cells; then PLGA devices were designed and tested in a diffusion chamber, and

finally the bioactivity of the released media was evaluated by measuring axon growth in DRG

cells exposed to the media for 72 hours. The combined drug delivery nerve guide was able

release FK506 for 20 days at concentrations (1-20ng/mL) that were shown to enhance DRG axon

growth. Furthermore, the released FK506 was bioactive and able to enhance DRG axon growth.

The combined drug delivery nerve guide can release FK506 for extended periods of time and

enhance axon growth and has the potential to improve nerve regeneration following a peripheral

nerve injury.

Keywords—*Peripheral nerve injury; FK506; nerve conduit; drug delivery*

Introduction

The extensive network of nerve tissue throughout the body makes peripheral nerves prone to external trauma related forces (1). Traumatic incidents such as motor vehicle accidents, crush injuries, or lacerations can cause peripheral nerve injuries resulting in impaired muscle function and loss of sensation (1). Peripheral nerve injuries frequently result in paralysis, chronic pain and neuropathies leading to severe disability in patients (2). Injuries to peripheral nerves can be very sharp and localized or can involve a segment of nerve. For injuries that result in a gap in the nerve, bridging strategies must be utilized. Nerve autograft has been the gold standard of treatment for such injuries (3). This involves the harvesting of donor nerve tissue and implanting the graft across the nerve gap (2). Nerve autografts have been generally successful but there are still problems associated with donor tissue availability (1), obtaining a donor nerve via single or multiple surgeries, scarring at donor and recipient sites, neuroma formation and the limited success at repairing gaps larger than 10mm (1). These issues led to the fabrication of hollow tube-like nerve guiding conduits to span the gap and provide structural support to the regenerating nerve (4). There are several FDA approved nerve conduits used clinically such as NeuroTube©, NeuroFlex©, NeuroMatrix©, NeuroLac© and NeuroGen© (4). These conduits are fabricated using collagen, polyglycolic acid (PGA) and poly lactide-co-caprolactone (PCL) (Kehoe et al., 2012). Other types of polymers such as poly lacto-glycolic acid (PLGA), polyurethane (PU), poly ethylene glycol (PEG) and chitosan are being investigated as potential nerve guides (3). These nerve guides, though structurally helpful, do not provide the necessary environment for the regenerating nerve (4). These deficiencies have prompted researchers to improve upon the current nerve conduit design (3). Following a peripheral nerve injury, axons will tend to regrow and cross short gaps, but they need guidance and the ability to overcome the

scarring process (5). To improve the repair process, in addition to providing guidance, therapeutic agents need to be provided at the site of transection or injury (6). Several drugs and growth factors have been shown to be effective in enhancing neurite outgrowth across nerve gaps (6). A study of nerve growth factor (NGF) loaded microspheres has shown potential in repairing nerve gaps (7). Collagen tubes loaded with NGF alone, or in combination with GDNF (glial cell line derived neurotrophic factor), have demonstrated neurite elongation *in vitro* (8). Approved by the FDA in 1994 for liver transplants, FK506 is one of the main systemic immunosuppressants used to prevent nerve allograft rejection (9). This drug has also been observed to have neuroregenerative properties when administered after peripheral nerve injuries (10). There are numerous in vivo small animal studies that have demonstrated improved rates of nerve regeneration with systemic delivery of FK506 (11-16). FK506 has been shown to enhance nerve regeneration in the setting of axotomy, autograft repair, and allograft repair. Treatment with FK506 has been reported to enhance the rate of axonal regeneration in the rat after nerve grafting into the injured spinal cord (17). FK506 has been useful to enhance regeneration after surgical repair by improving the rate of axonal growth with autografts (13, 14, 18). Despite the ability of FK506 to assist in nerve regeneration, systemic delivery has numerous potentially serious side effects such as central nervous system toxicity, infection, nephrotoxicity, and hyperglycemia (19-21). Local delivery of FK506 from a nerve guide could provide the neurotrophic benefits of FK506 on nerve regeneration but prevent the negative consequence of systemic delivery. The overall goal of this project is to design a drug delivering nerve conduit capable of controlled local delivery of bioactive FK506. We hypothesize that the drug-delivering PLGA nerve conduit will release bioactive concentrations of FK506 resulting in improved neurite outgrowth from DRG cells in vitro.

Materials and Methods

DRG explant culture

Fertilized chicken eggs (Merrills Poultry, ID) were incubated at ~39°C under 100% relative humidity for 12 days. The eggs were first cleaned with 70% ethanol and then opened to collect the embryos. Dorsal root ganglions were dissected from the embryos under a stereomicroscope using a standard dissection procedure (22). DRGs were separated carefully from connective tissue for culturing in 24-well plates coated with laminin (1 µg/ml). 500 µl of DMEM F12 medium supplemented with 10% FBS and 1% of Antimyotic/Antibiotic solution was added to each well. DRGs were plated at a density of one per well. DRG cultures were maintained in a humid atmosphere at 37°C and 5% CO_2 for 72 h. Unless specified otherwise, all reagents for cell culture were purchased from Fisher Scientific (Pittsburgh, PA).

Quantification of neurite outgrowth

After 72 hours in culture, DRGs were fixed in methanol, imaged using a widefield microscope (Nikon Spinning disk) with a phase contrast lens, and images captured with a digital camera at 4x magnification. Phase contrast images of DRGs were taken after 3 days of incubation, and the average neurite length was measured. Neurite elongation measurements were done using a previously discussed procedure (23). Briefly, the area of the ganglion body (A_{DRG}) and the total area of the DRG with the growing axons (A_{tot}) were measured using ImageJ 1.31v software (National Institutes of Health, Bethesda, USA). The average neurite length (l_{avg}) was calculated by: $l_{avg} = (A_{tot}/\pi)^{1/2} - (A_{DRG}/\pi)^{1/2}$ (23).

FK506 Dosing

An FK506 dosage curve was obtained to determine the optimal *in vitro* FK506 concentration for neurite growth. Four DRGs of similar size were used for each experiment and the experiments were repeated 3-4 times. Different set of DRGs were used for each repeat. The DRGs were treated with increasing concentrations of FK506 (Astellas Pharma, Northbrook, IL) (1, 5, 10, 20, 50 ng/ml). For control, DRG-explants were incubated in cell culture media matrix (DMEM+10% FBS+1% Antimyotic) without any growth factor or drug.

Device Design

Figure 1 shows the schematic diagram of the proposed device. The device consists of two concentric tubes and a reservoir in between the tubes that stores the drug. The drug is then released into the lumen, with diffusion controlled by the size and number of holes made on the inner conduit.

[Insert Figure 1]

Component Manufacturing

The stock 75/25 PLGA pellets (7525 DLG 7E, Evonik) were dissolved in acetone (10g PLGA to 20mL acetone) at 45°C. After manually stirring until a viscous solution, 6mL of ethanol was added to the solution. The ethanol serves as an emulsifier for the acetone to be displaced when the solution interacts with water. The solution was stirred at 180RPM on a stirring plate until visibly homogenous. The components were fabricated from PLGA emulsion in acetone and ethanol by solvent diffusion (24) and precipitation polymerization (25). Glass tubing was used as the mold for the inner and outer PLGA conduits. PLGA solution was suctioned into the glass molds and any excess PLGA was allowed to drip out of the molds. The conduits were then placed vertically into a water bath and allowed to cure. The water bath

quickly displaced the solvent and reduced deformations on the conduit caused by gravity. After

12 days, the conduits were released from the mold by using a matching ramrod to push them out.

Conduit dimensions were determined based on studies for sciatic nerve repair for rats. The inner

conduits were molded to dimensions of 2.4mm OD and 2mm ID and cut to a length of 13mm.

The outer conduit measured 4mm OD and 3mm ID and cut to a length of 7mm. End caps to join

the conduits and form the drug reservoir were manufactured by forming a PLGA sheet. The same

PLGA solution that was used for the conduits was poured on to a glass petri dish and left on a

45°C hot plate. This heating process cures the sheet in a bottom-up process that minimizes

wrinkling of the sheet as it expands and contracts. Following 4 hours on a hot plate, the sheet

was submerged into water, allowing the emulsion process to remove any remaining solvent. The

end caps were cut to size using a laser cutter.

Diffusion Hole

Diffusion holes of 120 μm were drilled into the inner conduits using laser milling with a

VLS3.60 CO_2 system (Universal Laser Systems). The laser holes were stabilized by pulsing the

laser multiple passes. This process annealed the PLGA surrounding the diffusion holes and made

them more stable once submerged into water.

Device Assembly

A solvent bonding process was adopted to assemble and seal the PLGA conduits. A

solution consisting of 2g PLGA and 2mL acetone was mixed together to form a viscous solution.

The solution was locally applied to join the end caps to the inner conduit. Following application

of solvent glue, the devices were placed in a water bath for 24 hours to remove any residual

solvents and return the assembly to a solid structural state. The assembled devices were sterilized

using Sterrad 100S system, chosen due to minimal degradation and deformation and verified

sterility. Following sterilization, FK506 was loaded by injecting through the outer conduit, the

injection hole was sealed using the same PLGA glue used during assembly.

PLGA Device Testing

Verification tests were performed to ensure that the nerve conduits released drug as

expected. The loaded devices were attached to the side wall of their respective receiver chambers

where tests were conducted. A 5mL transport tube was used as the receiver chamber for all of the

tests conducted. PDMS was used to attach the devices to the wall in a location that would still

allow for the devices to be fully submersed after the receiver chambers were filled with growth

media. Samples were collected from the receiver chamber at predetermined time frames. Sink

method was used for drug collection. Once the fluid was collected, the receiver chamber was

flushed and new medium was added to the chamber. After collection, samples were loaded into

96-well plates and amount of FK506 determined using an enzyme-linked immunosorbent assay

(ELISA) (Abnova, Taiwan). A release percentage was calculated by dividing the known released

drug mass by the total drug loaded into each device.

FK506-loaded Conduit Release Test

An FK506 release test was conducted in a simulated in-vivo environment. The conduits

used to perform this test were designed according to the size of a rat's sciatic nerve. The drug

reservoirs held approximately 20μL of drug and were loaded with an initial concentration of 0.05

mg/mL or 0.125 mg/ml of FK506 (Astellas Pharma). The test was conducted using eight devices:

three devices with 1x120μm diameter diffusion hole and loaded with FK506 concentration of

0.05 mg/ml, three devices with 1x120μm diameter diffusion hole and loaded with FK506

concentration of 0.125 mg/ml and two sealed devices loaded with 0.25 mg/ml of FK506 with no

diffusion holes. In addition to testing release kinetics, two varying initial concentrations were

tested to verify linear release scaling based on the amount of drug loaded.

These tests were conducted at ~37°C in a 5% CO_2 incubator under sterile conditions. The

devices were sterilized through the Sterrad 100S process and device loading and sealing was

done in a bio-sterile hood. The receiver chamber medium consisted of DMEM growth media

(Thermo Fisher, PA), 10% FBS (Thermo Fisher, PA) and 1% Antimyotic solution (Thermo

Fisher, PA) similar to what was used for DRG culture. The FK506 was diluted to the proper

initial concentrations using this media matrix. The ELISA kits used for this test accurately read

from 1-50ng/mL.

Bioactivity test

Drug released from the PLGA devices was used to verify and compare the bioactivity of

FK506 during the 20 day period. Release eluates from the devices were used for DRG explant

culture. 4 DRGs were tested per release sample and the test was repeated two times. DRGs were

plated at a density of one per well. DRG cultures were maintained in a humid atmosphere at

37°C and 5% CO_2 for 72 h using the same protocol described previously.

Statistical Analysis

The data were statistically analyzed by a one way ANOVA followed by a post-hoc test.

The outcome variable was neurite length, specific doses of FK506 and release eluates were

included in the test as indicator variables. Values with $P < 0.05$ were considered significant.

Results

[Insert Figure 2]

The device is composed of poly lactide-co-glycolide (PLGA) (75:25) to locally release FK506 (Figure 2). The main device components are as follows: An outer hollow PLGA tube of 3 mm ID and 4 mm OD defines the drug reservoir (Figure 2). A second inner PLGA tube of 2 mm ID 2.4 mm OD defines the guidance tube. The space between the inner and outer tubes defines the reservoir volume which stores FK506. A laser machined hole of diameter 120 μm in the inner tube (Figure 2) is created to define the diffusion area for drug to transport from the drug reservoir to the conduit lumen.

FK506 Growth

[Insert Figure 3]

An FK506 dosage curve was obtained to determine the *in vitro* concentration required for optimal neurite outgrowth on the DRG cells. The concentration range of 1-20 ng/ml was found to produce significant neurite growth in DRGs as compared to the control ($p < 0.05$). Figure 3 shows the images of DRGs and a plot of neurite outgrowth against different FK506 dosages. FK506 was observed to induce neurite elongation in the lower concentration range, with DRGs exposed to 1 ng/mL, 5 ng/mL, 10 ng/mL and 20 ng/mL FK506 having statistically longer neurite extensions than 0 ng/mL ($p < 0.05$) (Figure 3). In contrast there was no difference in neurite length between DRGs exposed to 50ng/mL FK506 or control.

Device Release

[Insert Figure 4]

Figure 4 shows the cumulative FK506 released from the different devices over the 20 day study period following the 24 hr flush. It shows that all the devices have 70% or more FK506 remains in the reservoir at the end of the experiment, indicating that sufficient drug is available in the device for a longer release if necessary. The device design goal was to release FK506 at a concentration between 1 and 20ng/mL, as suggested from the FK506 dosage curve results (Figure 3). There was a burst release in all devices tested, with approximately 10% released from the devices in the first 24 hours. The data for burst release is not shown on the graph. This burst may be the result of excess drug that was left on the outside of the device when filling or drug quickly passing through the hole during filling or between the fill time and the beginning of the experiment. This initial drug release was then washed away after replacing the media of the receiver chamber several times.

[Insert Figure 5]

Figure 5 shows local FK506 concentration in the receiver chambers for PLGA devices in the 20-day release study. Devices 1a-1c (0.05mg/mL FK506 and 1x120 μm hole) had the best *in vitro* FK506 release concentration, with the average starting concentration of 12 ng/mL in the day 1 collection, followed by 15, 10, 12, 11 and 10 ng/mL on the day 3, 5, 7, 11 and 15 respectively. The release kinetics of devices 1a-1c suggest that loading the conduit with a concentration of 0.05 mg/mL FK506 is able to maintain a FK506 concentration within the bioactive range for 15 days. Devices 2a-2c (0.125mg/mL FK506 and 1x120 μm hole) were also able to release FK506 at a concentration within target for the first 15 days, with the average starting concentration of 25ng/mL in the 1st day collection, followed by 21, 17, 15, 18 and 16 ng/mL on day 3, 5, 7, 11 and 15 respectively. The sealed devices 3a-3b (0.25mg/mL FK506 and no diffusion hole) had very little FK506 release in the first 3 days with the average starting

concentration of 1 ng/mL, followed by 0.5 and 1 ng/mL and dropped off to 0.03 ng/ml in the later collections, verifying that the reservoir was sealed and adding confidence that the drug collected from the receiver chamber was released through the inner conduit diffusion holes.

Therefore at simulated in vivo temperature and sterilized conditions, the nerve conduits deliver FK506 within an optimal range.

Bioactivity

[Insert Figure 6]

[Insert Figure 7]

It is not only important for the device to release FK506 in a controlled manner at a desired concentration but the released drug also must be bioactive. In order to evaluate the bioactivity of the released FK506 we exposed DRG cells to the collected release media and evaluated DRG neurite extension after 72 hours. We evaluated media collected on days 1, 7 and 15 from the release tests. As a reference we also exposed DRG cells to varying concentrations of fresh FK506 (0, 1 and 10 ng/mL), as shown in Figure 6,7. The FK506 release collected from devices 1a, 1b, 2a and 2b were chosen for these experiments. Overall, the results showed that the released FK506 still retained bioactivity, as DRGs exposed to released or fresh FK505 had enhanced growth compared to the control group ($p < 0.05$).

The media released from device 1a on day 1 (8.8 ng/ml), day 7 (9.5 ng/ml) and day 15 (8.2 ng/ml) and device 1b on day 1 (15.2ng/ml), day 7 (14.5 ng/ml) and day 15 (11.8 ng/ml) exhibited enhanced growth compared to a control media with no FK506 and, with an average neurite extension of 697.2 ± 25.3, 688.7±35.3 µm and 631.3 ±49.1 µm, respectively ($p < 0.05$) (Figure 6,

7). The released media collected from the devices exhibited similar levels of neurite growth when compared with 1-20 ng/mL of freshly prepared FK506 (p>0.05). Device 2a and 2b also showed positive results for FK506 bioactivity, as shown in Figure 7. The released FK506 from the 7 and 15 day collections was associated with 671.7±29.9 µm and 612.2±51.2 µm neurite outgrowth and was statistically different from the control group (p<0.05) (Figure 7). These results suggest that the FK506 released from the devices in the period from days 1-15 can promote neurite growth in chick DRGs, and the results from this device are closer to the optimal results obtained from the FK506 dosage curve.

The main outcomes of this study revealed that: (i) a PLGA drug delivery conduit was designed that could release optimum FK506 concentration (1-20 ng/mL) locally and (ii) the released FK506 was found to be bioactive and enhance DRG outgrowth.

Discussion

Many groups have utilized hydrogels, fibrin, and polymer gels as carriers with growth factors and small molecules inside the lumen of nerve conduits (3, 4). Attempts have also been made to incorporate drug into the biodegradable construct of the conduit itself. Each of these approaches has variable drug release kinetics with a burst of drug release that is neither sustained nor at the desired dose as the conduit degrades.   In addition, the use of various drug delivery mechanisms, such as microspheres, coatings or crosslinked polymers suffers from a lack of flexibility in choosing various drugs and drug concentrations when used with different types of peripheral nerve repair surgery. The shortcomings of current drug-delivering conduits in terms of burst effect, non-uniform dosage and uneven drug delivery necessitate a new approach to deliver drug for nerve regeneration. Our proposed localized drug delivery approach is unique in

that the desired drug is released from an integrated biodegradable reservoir through the hole into the lumen of the conduit. Our device design allows for easy modification of drug choice, concentration, or released volume by altering basic design parameters such as reservoir volume or diffusion hole size. Since our device doesn't rely on polymer degradation for drug release, we are able to easily change delivery parameters for different drugs and minimize initial burst release.

In conclusion, this study shows that our device can release optimal level of bioactive FK506 at a controlled rate for 20 days. The FK506 dosage curve sets the *in vitro* FK506 concentration requirement of 1-20 ng/mL for optimal neurite outgrowth of DRG cells. PLGA devices loaded with different FK506 concentrations were tested, and the majority of the devices met the requirement to maintain an optimum *in vitro* release concentration for a period of 20 days. Release samples were tested for bioactivity, and the neurite outgrowth of the DRG cells confirmed that the released drug (FK506) was still active on the 20th day of the release study. This release study also indicates that more than ~70% of the drug is still available in the device for further release.

This innovative device has the potential for broad applicability. In the future, this device could be used to deliver multiple drugs by creating separate drug reservoirs and diffusion areas. These could include growth-factors, small molecules, and synthetic and natural proteins. Our device also has the potential to function synergistically with stem cells or Schwann cell transplants by delivering proteins or growth factors to the cells to direct differentiation or function. This device could be used in conjunction with composite tissue allotransplantation, such as hand or face, to ensure robust nerve growth and functional recovery. This approach allows for easier customization to change the type of drugs and dosage of individual drugs

without devising a completely new biomaterial-drug conjugate each time. Our future aim is to evaluate the *in vivo* efficacy of this device to prevent allograft nerve rejection and potentially enhance nerve repair through the local delivery of FK506.

Acknowledgements

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Line numbers (left margin): 1 through 65.

Figure Legends

Figure 1 (a) Schematic of the drug delivery device demonstrating structural characteristics including: Inner (light blue) and outer (grey) PLGA conduit, drug reservoir (space between the conduits), and end caps defining boundary of reservoir (dark blue.) The diffusion hole (in the inner shell) can be modified to control the rate of diffusion.

Figure 2 (a) Conduit Components - inner conduit, outer conduit, and end caps (b) Inner conduit with two end caps defining the outer boundary of the reservoir (top) Completed PLGA device with inner and outer tube, as well as a diffusion hole on the inner conduit that enables the release of FK506 from the reservoir into the inner chamber (bottom) (c) Image of a ~124μm laser drilled hole in the inner conduit (d) Device setup in the receiver chamber

Figure 3 (a) Phase contrast images of DRGs treated with different concentrations of FK506: control 0 ng/ml (left), 1 ng/mL (center), 10 ng/mL (right) (b) Plot showing effect of standard FK506 dosages on DRG neurite elongation. Scale bar 500μm (*indicates statistically different from 0 ng/mL; $p<0.05$)

Figure 4 Cumulative FK506 release from drug delivering conduits over a period of 15 days. It shows that all the devices have released ~20% or more by the end of the experiment, suggesting that the release can be carried further. Plot shows % cumulative release for 1x120 μm hole device loaded with 0.05 mg/ml of FK506 (n=3) (blue line); 1x120 μm hole device loaded with 0.125 mg/ml of FK506 (n=3) (red line); device with no hole loaded with 0.25 mg/ml of FK506 (n=2) (green line).

Figure 5 FK506 release concentration in the receiver chamber over a period of 15 days *in vitro*. Plot shows average release concentration (ng/ml) for 1x120 μm hole device loaded with 0.05

mg/ml of FK506 (n=3) (blue line); 1x120 µm hole device loaded with 0.125 mg/ml of FK506

(n=3) (red line); device with no holes loaded with 0.25 mg/ml of FK506 (n=2) (green line).

Figure 6 Bioactivity of FK506 release collected from the devices. Bar graph shows average

neurite elongation (µm) of DRGs treated with FK506 released from devices 1a and 1b compared

to DRGs treated with freshly prepared FK506 (Control groups). Scale bar 500µm. (* indicates

statistically different from 0 ng/ml; $p < 0.05$)

Figure 7 Phase contrast images of DRGs treated with bioactive drug released from device 1a: (a)

day 1 release (8.8 ng/ml), (b) day 7 release (9.5 ng/ml), and (c) day 15 release (8.2 ng/ml). Phase

contrast images of DRGs treated with bioactive release from device 2a: (d) day 1 release (21.6

ng/ml), (e) day 7 release (13.6 ng/ml), and (f) day 15 release (14.3 ng/ml)

Figure 1

Diffusion hole

Outer PLGA Shell
Reservoir end caps
Nerve
Inner PLGA Shell
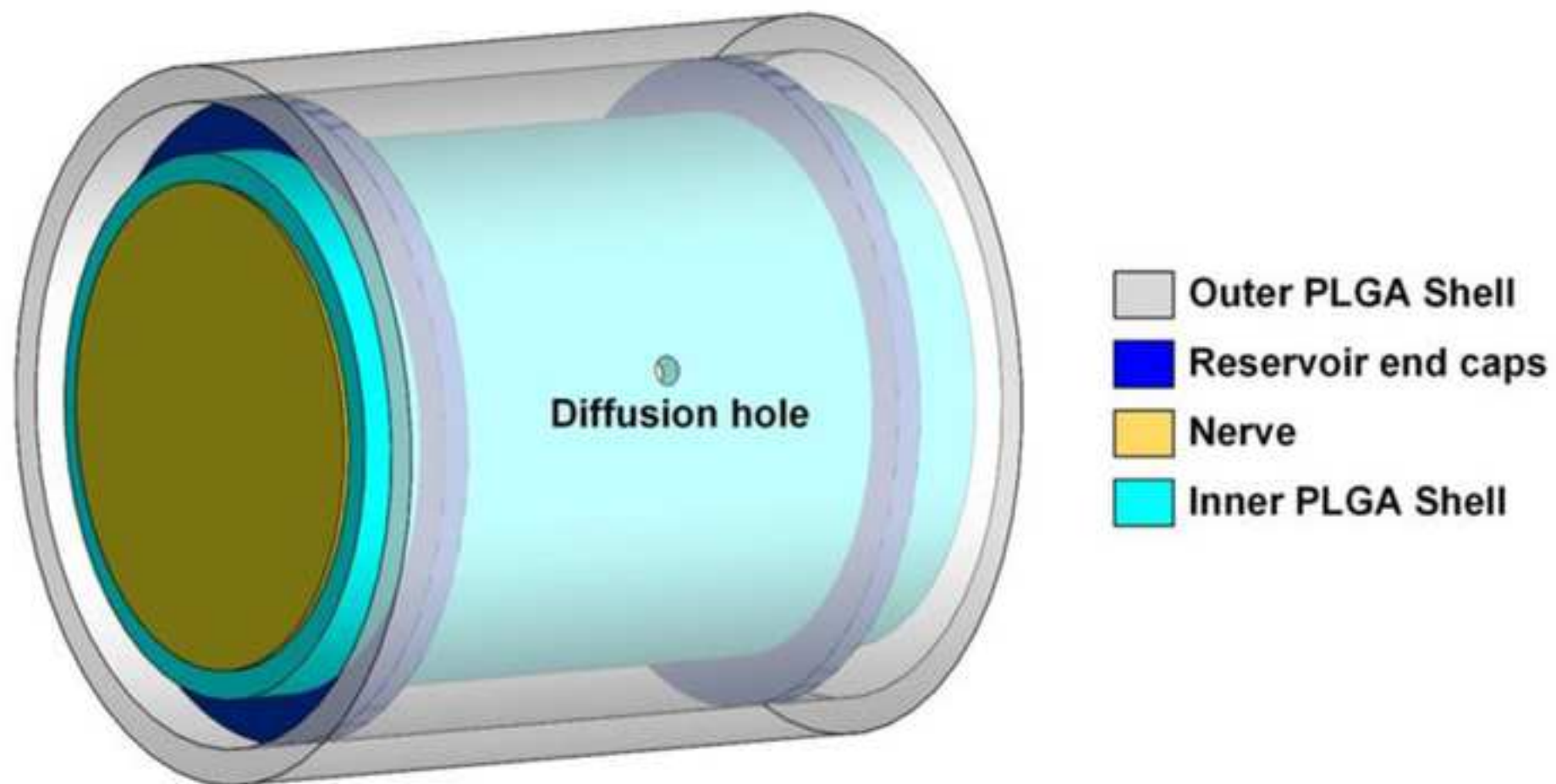
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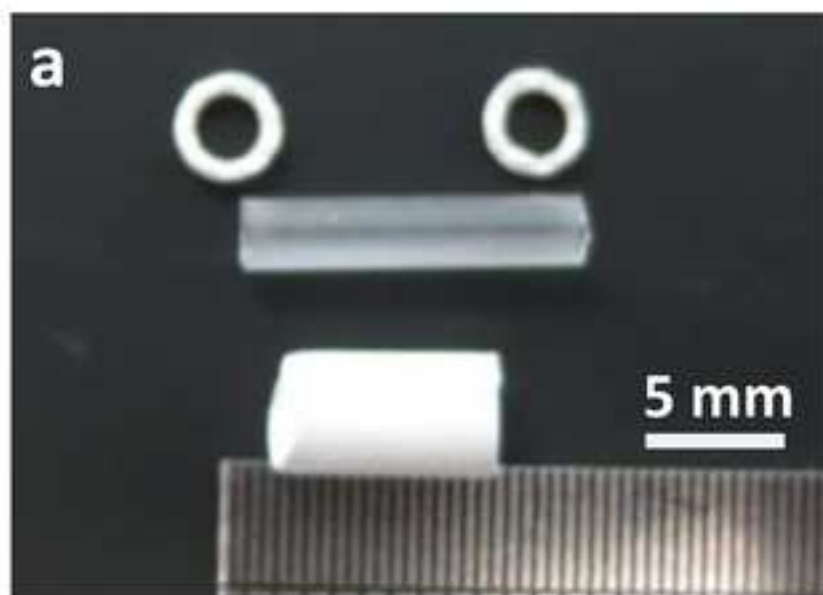
Click here to download Figure Figure_2.tif
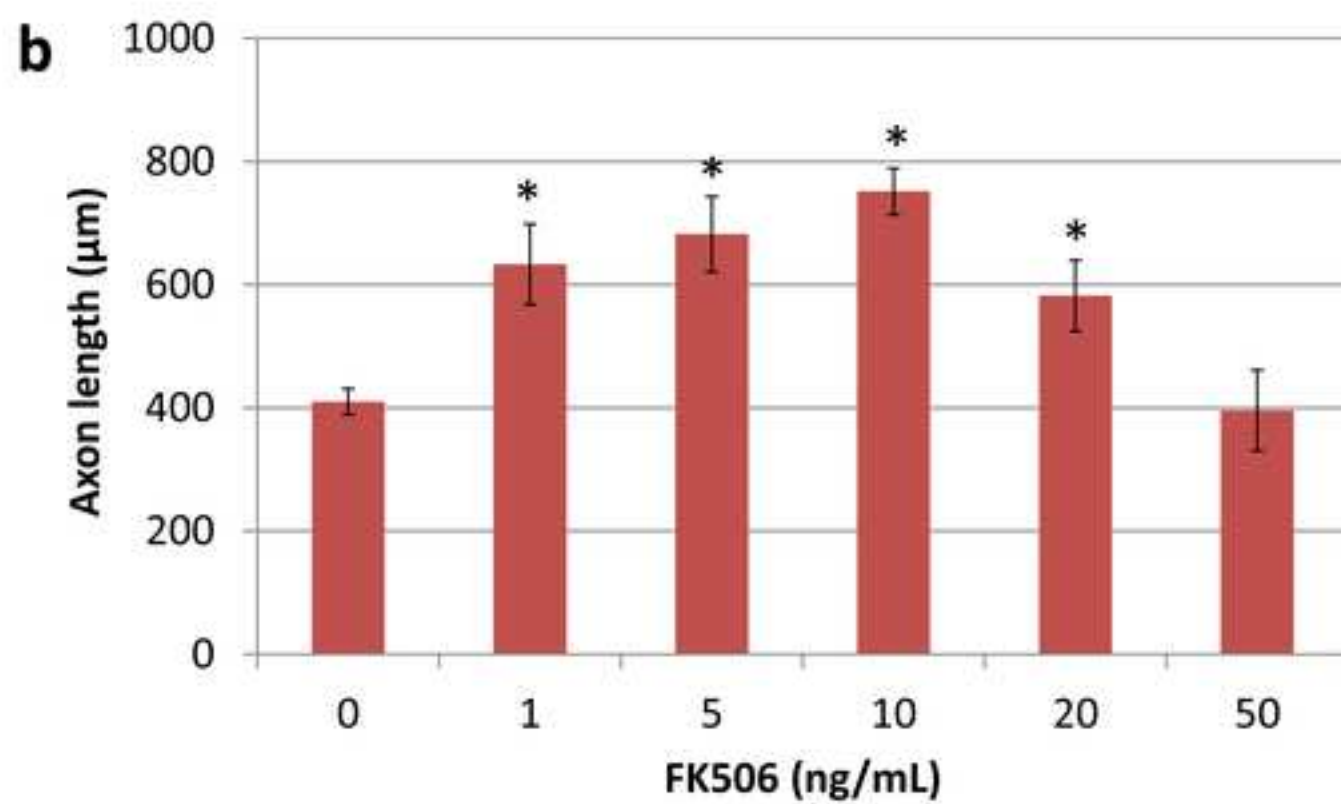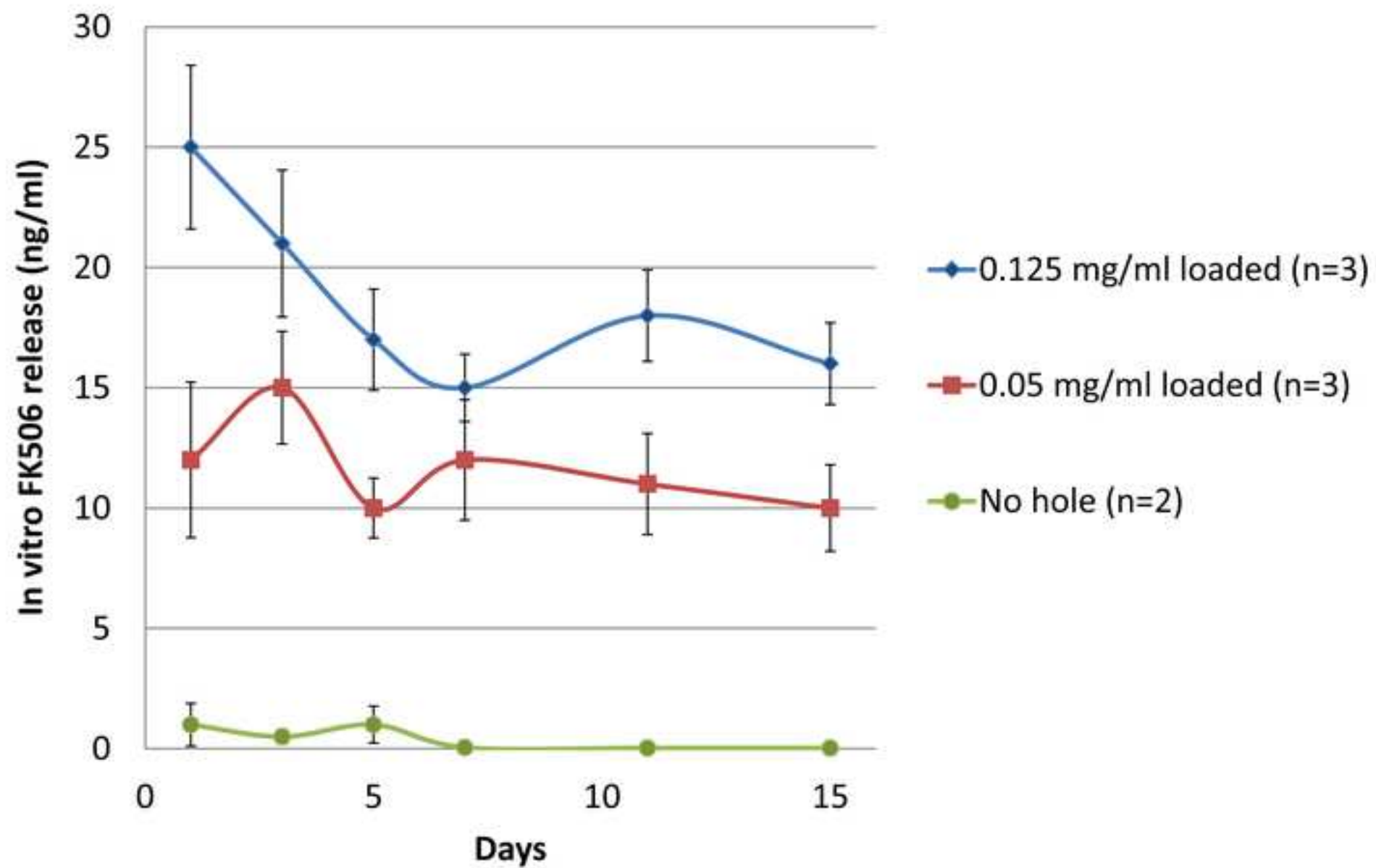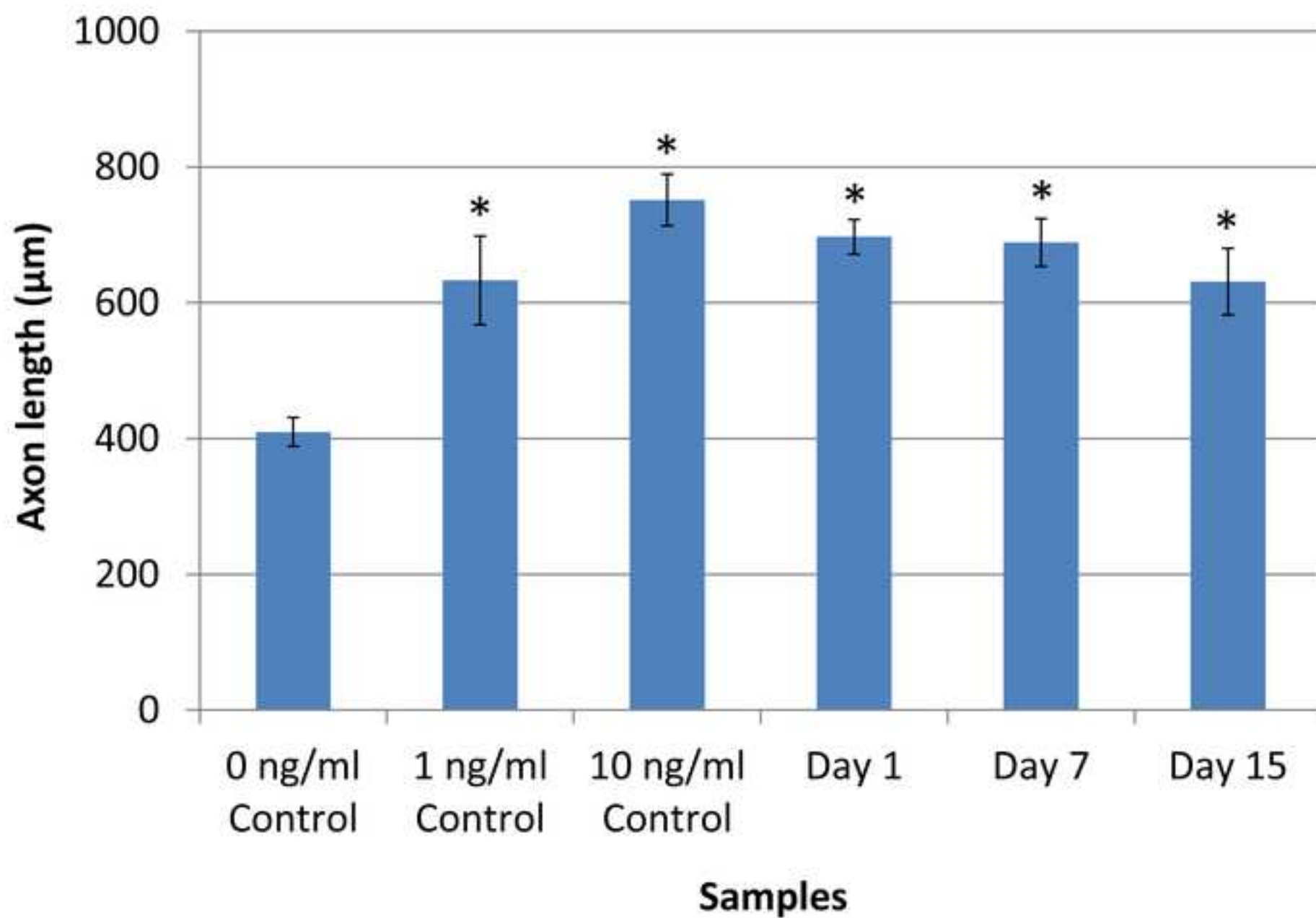

5 mm

Figure 3

Click here to download Figure Figure_3.tif
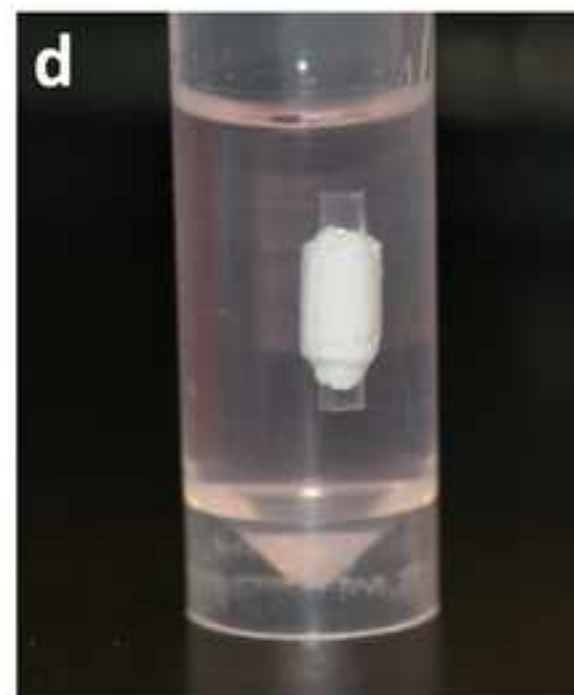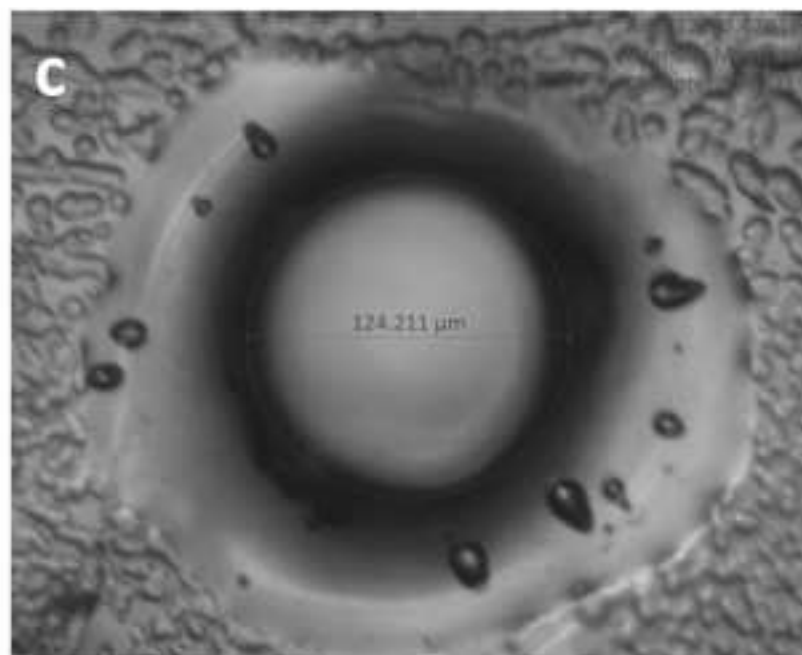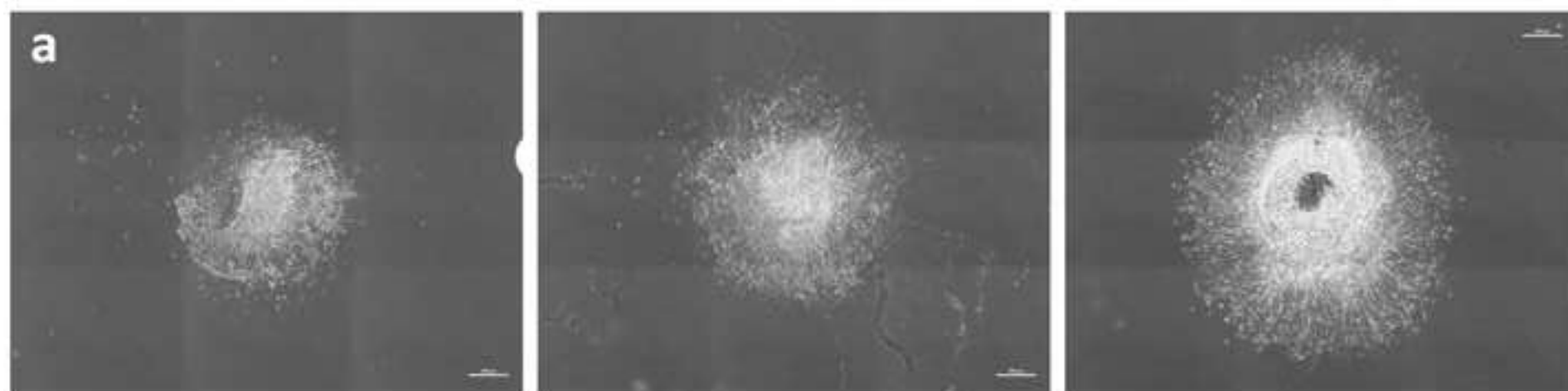
Figure 4

Figure 4

Figure 5

Figure 5

Figure 6

Figure 6

Figure 7